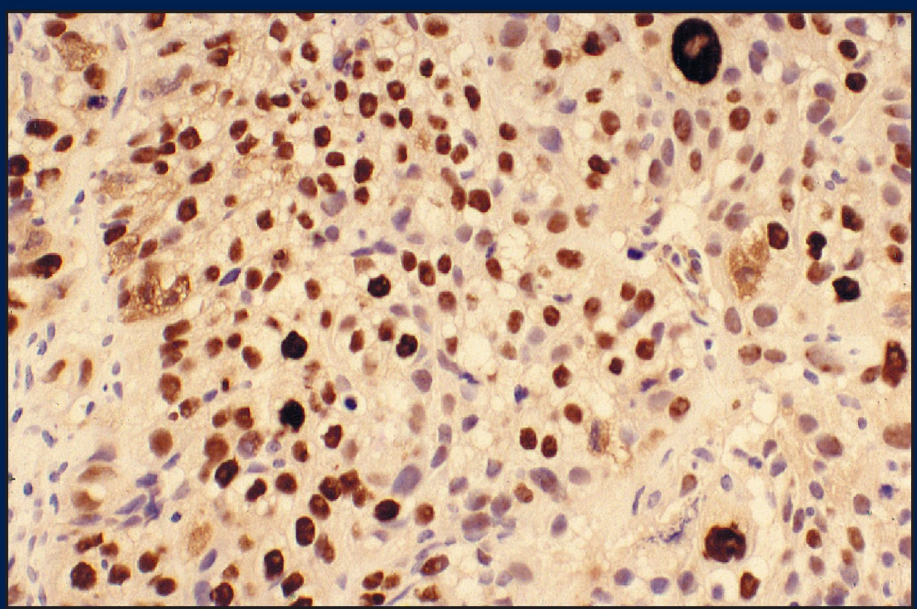
THE ROLE OF ONCOGENES IN THE RADIATION RESPONSE OF HUMAN TUMOURS

In Vitro and Clinical studies



J. Pomp

THE ROLE OF ONCOGENES IN THE RADIATION RESPONSE OF HUMAN TUMOURS

J. Pomp

THE ROLE OF ONCOGENES IN THE RADIATION RESPONSE OF HUMAN TUMOURS.

In Vitro and Clinical studies

Cover: histology of a p53 tumour as stained by DO7

THE ROLE OF ONCOGENES IN THE RADIATION RESPONSE OF HUMAN TUMOURS.

In Vitro and Clinical studies

Een wetenschappelijke proeve op het gebied van de

Medische Wetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Katholieke Universiteit Nijmegen,

volgens besluit van het College van Decanen

in het openbaar te verdedigen op

donderdag 29 maart 2001

des namiddags om 1.30 uur precies

door

Jacquelien Pomp

Geboren op 28 februari 1958 te Willemstad, Curaçao

Promotor:	Prof. Dr. J.W.H. Leer
Co-promotor:	Dr. J. Wondergem (Leids Universitair Medisch Centrum)
Manuscriptcommissie:	Prof. Dr. A. J. van der Kogel
	Dr. A. Begg (Nederlands Kanker Instituut)
	Prof. Dr. C. Punt

This thesis was supported by grants from the committee for scientific research and the directory board of the Reinier de Graaf Group

CONTENTS Page

Chapter 1	Introduction and aim of the study	7
Chapter 2	The Influence of the oncogenes *NRAS* and MYC on the Radiation Sensitivity of Cells of a Human Melanoma Cell Line *Radiation Research 146: 374-381 1996*	25
Chapter 3	The influence of the oncogene *NRAS* on cell cycle delay in a human melanoma cell line with reduced radioresistance *Oncology Reports, 7: 663-667, 2000*	45
Chapter 4	Radiotherapy for oesophagus carcinoma- the impact of p53 on treatment outcome *Radiotherapy and Oncology 46 (2); 179-184 1998*	57
Chapter 5	E-cadherin expression in oesophageal carcinoma treated with high-dose radiotherapy; a correlation with pre-treatment parameters and treatment outcome *Journal of Cancer Research and Clinical Oncology 125 (11); 641-645 1999*	75
Chapter 6	p53 and radiotherapy for oesophagus carcinoma; a comparison between 4 different antibodies *Oncology Reports 7: 1075-1078, 2000*	87
Chapter 7	Cell density dependent plating efficiency affects outcome and inter-pretation of colony forming assays *Radiotherapy and Oncology 40: 121-125 1996*	99
Chapter 8	Summary and discussion / Samenvatting en discussie	111
	Dankwoord /Acknowledgements	123
	Curriculum Vitae	125
	List of Publications	127

CHAPTER I

INTRODUCTION AND AIM OF THE STUDY

Introduction

Background

Radiotherapy is one of the four major treatment modalities for cancer. The others are surgery, chemotherapy, and to a lesser extent, immunotherapy. Hormonal manipulation of tumours, although not considered a treatment leading to direct kill [60], is applied as adjuvant therapy or palliatively for breast or prostate cancer. Radiotherapy can be used as a single modality treatment or combined with others. Radiotherapy offers excellent results not only for cure, but also in palliation (pain, bleeding, dyspnoe and spinal cord compression) [9,67].

In radiotherapy, the limiting factor is the maximal dose which is tolerated by the normal tissue. In multiple disciplines ranging from radiation physics to radiobiology and molecular biology, mechanisms are being investigated, how tumours can be eradicated with as little damage to normal structures as possible. Progress in physics allows higher tumour doses to be delivered with greater accuracy. Radiobiologists and molecular biologists study the response of the tumour and normal tissues following treatment with ionising radiation and the determinants of intrinsic radiosensitivity. Progress in unravelling these mechanisms may allow modifying the radiation response of tumours and normal tissue, ultimately increasing the therapeutic ratio.

Radiobiological principles.

The success of radiotherapy is dependent on several radiobiological parameters. Firstly, the intrinsic sensitivity of cells comprising both tumours and normal tissues to ionising radiation is important. Secondly, the response of the tumour is also influenced by tumour bed characteristics e.g. oxygenation and temperature, which influence the fixation of radiation damage. Thirdly, the total tumour load, the number of clonogenic cells (i.e. the dividing cells, responsible for growth of the tumour) that has to be killed, determines the chance of achieving local tumour control [14].

The cellular response to ionising radiation determines the intrinsic radiosensitivity. This response consists of a cascade of reactions; the induction of DNA damage, single strand breaks (ssb), double strand breaks (dsb), loss or alteration of bases and cross-linking between DNA strands. Next the recognition of DNA damage followed by gene expression [72,88], the induction and/or recruitment of proteins and enzymes involved in repair of the DNA damage and the initiation of the machinery necessary to stop the cell cycle in order to give time for DNA damage repair [52,58]. Furthermore, radiation initiates a chain of reactions not only in the nucleus but also in the cell membranes [11,23,34,41,53].

Intrinsic radiosensitivity can be determined by a variety of *in vitro* and *in vivo* assays. Clonogenic assays form the basis to establish survival curves and rank tumours according

to their radiosensitivity. The linear quadratic (LQ) model helps to estimate radiobiological effectiveness of different dose-fractionation schedules with regard to acute and late toxicity. Its formula contains parameters specific for a cell line or tissue and treatment-parameters. [8,21,28]. The α-component represents the unrepairable DNA damage and determines the first part of the survival curve, while the β-component is related to the DNA damage that can be repaired and this component shapes the second part of the survival curve.

Cell cycle effects after radiotherapy.

Cells that grow and divide pass through a number of events, called the cell cycle. The cell cycle consists of four phases (Figure 1). The first part of the cycle is the G_1 phase, followed by the S(ynthese) phase. During the S phase DNA is replicated; an exact copy of the whole genome is created in order to form two identical daughter cells. The next phase is the G_2 phase and division takes place during the M(itose), where-after cells enter a new cycle (the next G_1 phase). The G_2 phase prepares the cell for the mitosis. During the mitosis the duplicated DNA strands are equally divided over the two new identical cells.

Several enzymes mediate the transition from G_1 to S and from G_2 to M. The S phase Promoting Factor (SPF) is responsible for the transition from G_1 to S while the Mitosis Promoting Factor (MPF) thrives the cell from the S phase into the M phase. These enzymes consist of a catalytic subunit, the cyclin dependent kinase (cdk), and a cyclin. Cyclins are essential for the kinase activity of the cdk and their expression is regulated throughout the cell cycle. Cdk levels are constant through the cell cycle. Cells will only enter the next phase when all events in the previous phase have been completed. Cyclins are responsible for the correct order of events regulating the phases of the cell cycle.

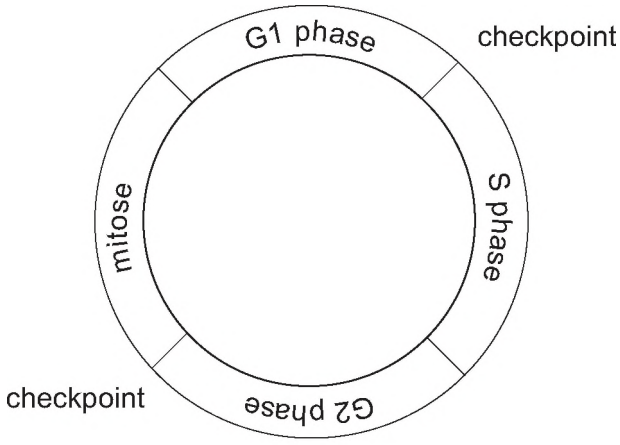


figure 1

For an adequate repair of radiation-induced DNA damage, it is necessary that this damage be recognised before division. Next, cell cycle progression and S phase should be slowed down in order to allow for DNA repair [46,52,58]. The biochemical pathway responsible for adequate recognition of DNA damage and the consequent machinery exists of two checkpoints. These operate at the end of the G_1 and G_2 phase respectively; the so-called G_1 and G_2 checkpoints [46]. These checkpoints may be interrelated [71].

The G_1 checkpoint is responsible for G_1 arrest, facilitating DNA repair. p53 is essential in the regulation of the G_1 checkpoint (see below) [10,15,27,29,49,50,54].

The G_2 checkpoint checks the quality and quantity of DNA before entry into mitosis. If DNA damage is present, the duration of the G_2 phase is prolonged. Prolonged G_2 delay can therefore also be seen as an expression of DNA damage [68]. To accomplish a G_2 delay, a complex interaction of cyclins and cyclin dependent kinases is necessary [46]. p53 is also involved in the G_2 delay (see below) [40,77,90]. Inactivation of cyclin B1-associated cdc2 kinase is essential in the p53-mediated G_2/M arrest [47,70]. Based on current literature no clear correlation between G_2 delay and radiosensitivity was found [68]. Extended G_2 delay may lead to increased radioresistance as it allows for longer repair before division. Prolonged G_2 delay may however also be correlated with a more radiosensitive phenotype as it may reflect insufficient repair damage before mitosis. If the G_2 delay itself is involved in DNA damage repair before mitosis, then a defect in G_2 delay will result in increased kill -mitotic catastrophe- due to unrepaired damage. These issues still need to be resolved.

The S phase delay is mediated by targeting proteins that are responsible for the DNA replication. p53 is not required for this. Proteins involved in initiation of DNA replication and elongation are part of the S phase machinery. It is suggested that the ATM gene is involved in the regulatory pathway to control DNA replication. AT (Ataxia Teleangiectasia) cells, with an inherited high intrinsic radiosensitivity, due to an aberrant ATM gene, fail to inhibit replicon initiation and chain elongation as a response to ionising radiation. The S phase delay is studied with much higher doses than the G_1 and the G_2 checkpoint and relative high doses are necessary to establish measurable effects. This makes the S phase checkpoint a less important determinant in the cell's radiosensitivity in clinical setting [46].

Oncogenes in Radiotherapy

Proto-oncogenes are normal cellular genes. Proto-oncogenes play a role in DNA damage recognition, the regulation of checkpoints, are often involved in DNA damage repair [25,43,45,89] and cell cycle control [26]. Therefore it is likely that they play a role in the response to DNA damage caused by ionising radiation [10,15,49].

When proto-oncogenes become activated or deregulated by means of mutation, amplification or increased expression, they are called oncogenes. Activated oncogenes play a role in the malignant development of cells. Tumour suppressor genes prevent malignant trans-

formation of the cells (carcinogenesis). When tumour suppressor genes have a deficit in expression, by mutation or deletion, a malignant tumour may develop. For this, both alleles of the tumour suppressor gene must be inactivated. This is the so-called "loss of function" mechanism because loss of function of the tumour suppressor genes contributes to tumour formation.

About a decade ago, reports in the literature appeared in which expression of oncogenes correlated with a radioresistant phenotype [6] suggesting a common background for malignant deterioration and altered radiation response. However, mechanisms of how oncogenes can affect the radiation response have not yet been fully elucidated in the year 2001.

The first attempt to investigate the influence of one single oncogene on radiation response was to compare cells that only differ in the expression of one oncogene. For these experiments, cells are stably transfected with the oncogene of interest. Next, experiments are performed in which the parental cell line serves as a control. Differences in sensitivity are then assumed to be solely due to the presence of the transfected oncogene. It should be kept in mind, however, that these tests do not explain "how" an oncogene influences radiation response: one oncogene can affect multiple pathways, which may all interfere differently with the response to ionising radiation.

The most popular oncogenes studied with regard to their influence on radiation response are *ras* and *myc* and the tumour suppressor gene p53.

The *myc* oncogene is involved in the normal proliferation, transformation and differentiation. It is a transcription factor and by itself not sufficient to transform cells. In normal cells the expression of *myc* is dependent on growth factors and essential for the progression through the cell cycle. It activates cell death by apoptosis (suicide of the cell) when over-expressed.

The *ras* proto-oncogene codes for the membrane protein $p21^{ras}$. The product of this gene has a molecular weight of 21 kDA. It belongs to the family of GTP-ases and is involved in the cell growth and differentiation. Whenever *ras* is over-expressed or mutated it acts as an oncogene, stimulates the activity of protein kinases and activates transcription of other genes like *c-myc*, *c-fos* and *c-jun*.

Transfections with *Hras* and *myc* oncogenes lead to increased radioresistance in rat embryonal fibroblasts [64,65]. This observation correlated with the stronger and longer suppression of DNA synthesis after radiation [86]. However, in other systems, such as Rat-1 cells and a human mammary epithelial cell line, transfected *ras* had no influence on radiosensitivity [3,37].

Besides the *myc* and *ras* oncogene, the p53 tumour suppressor gene has also been extensively studied with regard to radiation response. The p53 tumour suppressor gene encodes for a nuclear phosphoprotein of 53 kDa. The protein product of this suppressor gene, wild type p53, plays a role in cell cycle regulation and maintaining the integrity of the genome. p53 protein is stabilised following DNA damage, leading to upregulation. It then binds to

DNA in a sequence dependent manner and acts as a transcriptional activator [46]. The triggers for p53 induction after radiotherapy are DNA dsbs. Other toxic events may also cause activation of p53 dependent pathways, such as treatment with cisplatin, hypoxia or heat [35,38,74,80]. p53 regulates cell cycle arrest at the end of the G_1 phase [10,27,29,54] and is involved in the G_2 delay [20,42,55,76,84,87,90]. Besides cell cycle regulation, p53 is also responsible for apoptosis after radiotherapy or other cytotoxic treatments [13,17,49], although not always obligatory [62]. The ability to undergo p53-mediated apoptosis depends on the cell type [66]. Detection of DNA damage and subsequent suicide of the cell are also mechanisms to protect "damaged" cells from malignant transformation: cells with genetic defects are hereby eliminated [59,81]. Wild type p53 can sense DNA damage, or direct repair machinery to the sites of DNA damage [7,15,48,69,73]. It consequently leads to transcriptional activation of genes that contain a p53-DNA binding consensus sequence. The expression of $p21^{waf1/cip1}$ protein (wild type p53 activated fragment) is upregulated by p53 and this can inhibit the activity of cyclin D- and cyclin E-associated kinases [31,33,44].

Cells can die in two ways after cytotoxic treatment: mitotic death, or apoptosis. Apoptosis influences the low dose part of the survival curve [57]. There is no consensus whether apoptosis by itself determines clonogenic survival [4,63], as radiosensitivity may not be dependent upon the way cells die; mitotic death or apoptosis. A cell's rescue from apoptosis or mitotic death can increase clonogenic survival. Arends et al. [5] transfected *myc* and mutated *ras* in immortalised rat fibroblasts and found that mutated *ras* correlates with reduced apoptosis (due to absence of endonuclease activity, crucial for DNA fragmentation at apoptosis). This may be one explanation how *ras* in cells with wild type p53 can increase radioresistance. Vice versa, it has been suggested that when cells already have a reduced apoptotic response due to the presence of mutated p53, the addition of an oncogene like *ras* has less effect than in cell lines with wild type p53 (Chapter 3). The group of McKenna [75] also showed that inhibition of the *Hras* oncogene restored the apoptosis induction after radiotherapy in rat embryo fibroblasts. Some authors have suggested that p53 status could be a determinant for intrinsic radiosensitivity [13,56,57]. Although p53 plays an important role in the response to ionising radiation, up till now no clear correlation between radiosensitivity and p53 status can be established [15]. It may also be possible that the p53 status determines whether and how expression of a certain oncogene affects radiation response [1,24].

Another characteristic of p53 is its prognostic value with regard to the development of distant metastasis. Mutation in p53 is often predictive for a worse prognosis in a variety of tumours [12,19,39,51]. This may be due to an influence on local control of tumours, or by the increased propensity of the cells to metastasise. The better the local control, the more important becomes the prevention of distant metastases. Furthermore, reduction of apoptosis, as seen when p53 is mutated, has been reported to be associated with an increased risk of distant metastases [82].

In the seventies, it was suggested that radiation itself could influence the metastatic potential of tumours. However, most literature was descriptive and no molecular techniques were available to investigate which genes were involved in this phenomenon [85]. More recent literature has suggested a link between p53 and the cell adhesion molecule e-cadherin [16,18,22]. E-cadherin is a transmembrane glycoprotein that mediates the cell-adhesion and plays a role in the cell-cell contact of normal epithelium. Its presence can be detected by immunohistochemical techniques. Its gene maps to chromosome 16q22, and the product of this gene plays a role in embryogenesis and organogenesis by mediating epithelial cell-cell recognition and adhesion processes. Cadherins are bound to intracellular proteins, the catenins. E-cadherin expression can be altered or reduced in many tumours leading to detachment of cancer cells, facilitating the formation of metastases [30,36,78,79]. E-cadherin can be seen as a tumour suppressor gene [61] with prognostic value in prostate, colon breast and other tumours [12,19,39,51,83]. Very recently, *in vitro* studies have also found that e-cadherin is upregulated by ionising radiation, suggesting that radiotherapy may influence the formation of distant metastases via this pathway [2,32].

Aim of the study

This study focuses on cellular mechanisms defining the radiation response of tumour cells. The impact of oncogene expression on radiosensitivity in human tumours is investigated. Both *in vitro* assays, determining clonogenic cell survival, and clinical data were used to determine how the oncogenes *MYC* and *RAS* and the p53 status contribute to radiation response and outcome after radiotherapy.

For the *in vitro* assays, we used a melanoma cell line from human origin. Melanomas are known for their high radioresistance and often have mutations in the *NRAS* oncogene or overexpression of *MYC*. We studied whether expression of either oncogene could alter the radiosensitivity of the parental cell line. The next step was to determine cell cycle alterations in oncogene transfected cell lines in their response to ionising radiation.

For the clinical investigations, we used data of a group of patients with locally advanced oesophagus carcinoma who had been treated by high dose radiotherapy and followed prospectively. The impact of oncogene expression on the outcome of radiation treatment was determined.

The ultimate aim of this study is to see whether knowledge of oncogene activation could be a predictive factor for outcome of radiotherapy treatment, and whether it should be taken into account to determine the optimal treatment for patients with a malignant tumour.

The following questions served as the basis for the investigations and are discussed in each chapter separately.

- Do oncogenes *MYC* and *NRAS* alter the radiosensitivity of a human melanoma cell line in vitro?
- What is the effect of transfection with *NRAS* on cell cycle response after radiotherapy?
- Is there a correlation between p53 expression and the outcome of radiotherapy treatment of patients with oesophagus carcinoma?
- Is e-cadherin expression related to p53 status in a group of patients with oesophagus carcinoma and is e-cadherin of prognostic importance in tumours treated by radiotherapy exclusively?
- Is the choice of antibody in the detection of p53 expression of importance in a retrospective study?
- How does cell density dependent plating efficiency affect the outcome and interpretation of clonogenic assays?

Literature

1. Agapova, L.S., Ivanov, A.V., Sablina, A.A., et al. P53-dependent effects of ras oncogene on chromosome stability and cell cycle checkpoints. Oncogene 18: 3135-3142, 1999.

2. Akimoto, T., Mitsuhashi, N., Saito, Y., Ebara, T. and Niibe, H. Effect of radiation on the expression of e-cadherin and alpha-catenin and invasive capacity in human lung cancer cell line in vitro. Int. J. of Radiat. Oncol., Biol., Phys. 41: 1171-1176, 1998.

3. Alapetite, C., Baroche, C., Remvikos, Y., Goubin, G. and Moustacchi, E. Studies on the influence of the presence of an activated ras oncogene on the in vitro radiosensitivity of human mammary epithelial cells. Int. J. Radiat. Biol. 59: 385-396, 1991.

4. Aldridge, D.R., Arends, M.J. and Radford, I.R. Increasing the susceptibility of the rat 208F fibroblast cell line to radiation-induced apoptosis does not alter its clonogenic survival dose-response. Br. J. Cancer 71: 571-577, 1995.

5. Arends, M.J., Mcgregor, A.H., Toft, N.J., Brown, E.J. and Wyllie, A.H. Susceptibility to apoptosis is differentially regulated by c-*myc* and mutated Ha-*ras* oncogenes and is associated with endonuclease availability. Br. J. Cancer 68: 1127-1133, 1993.

6. Arlett, C.F., Green, M.H., Priestley, A., Harcourt, S.A. and Mayne, L.V. Comparative human cellular radiosensitivity: I. The effect of SV40 transformation and immortalisation on the gamma-irradiation survival of skin derived fibroblasts from normal individuals and from ataxia-telangiectasia patients and heterozygotes. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 54: 911-928, 1988.

7. Bakalkin, G., Yakovleva, T., Selivanova, G., et al. p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. Proc. Natl. Acad. Sci. U.S.A. 91: 413-417, 1994.

8. Barendsen, G.W. Dose fractionation, dose rate, and isoeffect relationships for normal tissue response. Int. J. Radiat. Oncol. Biol. Phys. 8: 1981-1997, 1982.

9. Bates, T. A review of local radiotherapy in the treatment of bone metastases and cord compression. Int. J. Radiat. Oncol. Biol. Phys. 23: 217-221, 1992.

10. Bernhard, E.J., Maity, A., Muschel, R.J. and McKenna, W.G. Effects of ionizing radiation on cell cycle progression. A review. Radiat. Environ. Biophys. 34: 79-83, 1995.

11. Berroud, A., Leroy, A. and Voisin, P. Membrane oxidative damage induced by ionizing radiation detected by fluorescence polarization. Radiat. Environ. Biophys. 35:289-295, 1996.

12. Bertorelle, R., Esposito, G., Del Mistro, A., et al. Association of p53 gene and protein

alterations with metastases in colorectal cancer. Am. J. Surg. Pathol. 19:463-471, 1995.

13. Blank, K.R., Rudoltz, M.S., Kao, G.D., Muschel, R.J. and McKenna, W.G. The molecular regulation of apoptosis and implications for radiation oncology [review]. Int. J. Radiat. Biol. 71: 455-466, 1997.

14. Brenner, D.J. Dose, volume, and tumor-control predictions in radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 26: 171-179, 1993.

15. Bristow, R.G., Benchimol, S. and Hill, R.P. The p53 gene as a modifier of intrinsic radiosensitivity -Implications for radiotherapy [Review]. Radiother. Oncol. 40: 197-223, 1996.

16. Bukholm, I.K., Nesland, J.M., Karesen, R., Jacobsen, U. and Borresen Dale, A.L. Expression of E-cadherin and its relation to the p53 protein status in human breast carcinomas. Virchows Arch. 431: 317-321, 1997.

17. Canman, C.E., Chen, C.Y., Lee, M.H. and Kastan, M.B. DNA damage responses: p53 induction, cell cycle perturbations, and apoptosis. Cold Spring Harb. Symp. Quant. Biol. 59: 277-286, 1994.

18. Cano, A., Gamallo, C., Kemp, C.J., et al. Expression pattern of the cell adhesion molecules. E-cadherin, P-cadherin and alpha 6 beta 4 intergrin is altered in pre-malignant skin tumors of p53-deficient mice. Int. J. Cancer 65: 254-262, 1996.

19. Casson, A.G., Kerkvliet, N. and O'Malley, F. Prognostic value of p53 protein in esophageal adenocarcinoma. J. Surg. Oncol. 60: 5-11, 1995.

20. Ceraline, J., Deplanque, G., Duclos, B., et al. Inactivation of p53 in normal human cells increases G_2/M arrest and sensitivity to dna-damaging agents. Int. J. Cancer 75: 432-438, 1998.

21. Chadwick, K.H. and Leenhouts, H.P. *The Molelcular Theory of Radiation Biology*, Berlin: Springer-Verlag, 1981,

22. Charpin, C., Garcia, S., Bouvier, C., et al. E-cadherin quantitative immunocytochemical assays in breast carcinomas. J. Pathol. 181: 294-300, 1997.

23. Cheng, A.C., Hogan, J.L. and Caffrey, M. X-rays destroy the lamellar structure of model membranes. J. Mol. Biol. 229: 291-294, 1993.

24. Chiarugi, V., Magnelli, L., Cinelli, M., Turchetti, A. and Ruggiero, M. Dominant oncogenes, tumor suppressors, and radiosensitivity. Cell Mol. Biol. Res. 41: 161-166, 1995.

25. Chung, D.C. and Rustgi, A.K. DNA mismatch repair and cancer. Gastroenterology 109:

1685-1699, 1995.

26. Coleman, W.B. and Tsongalis, G.J. Multiple mechanisms account for genomic instability and molecular mutation in neoplastic transformation. Clin.Chem. 41: 644-657, 1995.

27. Cox, L.S. and Lane, D.P. Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. Bioessays 17: 501-508, 1995.

28. Dale, R.G. The application of the linear-quadratic dose-effect equation to fractionated and protracted radiotherapy. Br. J. Radiol. 58: 515-528, 1985.

29. Di Leonardo, A., Linke, S.P., Clarkin, K. and Wahl, G.M. DNA damage triggers a prolonged p53-dependent G_1 arrest and long-terminduction of Cip1 in normal human fibroblasts. Genes Dev. 8: 2540-2551, 1994.

30. Dobson, H., Pignatelli, M., Hopwood, D. and D'Arrigo, C. Cell adhesion molecules in oesophageal epithelium. Gut 35: 1343-1347, 1994.

31. Dulic, V., Kaufmann, W.K., Wilson, S.J., et al. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G_1 arrest. Cell 76: 1013-1023, 1994.

32. Ebara, T., Mitsuhashi, N., Saito, Y., Akimoto, T. and Niibe, H. Change in e-cadherin expression after x-ray irradiation of a human cancer cell line in vitro and in vivo. Int. J. Radiat. Oncol., Biol., Phys. 41: 669-674, 1998.

33. el Deiry, W.S., Harper, J.W., O'Connor, P.M., et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 54: 1169-1174, 1994.

34. Floersheim, G.L. Calcium antagonists protect mice against lethal doses of ionizing radiation. Br. J. Radiol. 65: 1025-1029, 1992.

35. Fritsche, M., Haessler, C. and Brandner, G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents [published erratum appears in Oncogene 1993 Sep;8(9):2605]. Oncogene 8: 307-318, 1993.

36. Frixen, U.H., Behrens, J., Sachs, M., et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol. 113: 173-185, 1991.

37. Garden, A.S., Meyn, R.E., Weil, M.M., Lebovitz, R.M. and Lieberman, M.W. The influence of *ras* oncogene expression on radiation response in the Rat-1 cell. Int. J. Radiat. Biol 62: 307-311, 1992.

38. Graeber, T.G., Peterson, J.F., Tsai, M., Monica, K., Fornace, A.J., Jr. and Giaccia, A.J. Hypoxia induces accumulation of p53 protein, but activation of a G_1 phase checkpoint

by low-oxygen conditions is independent of p53 status. Mol. Cell Biol. 14: 6264-6277, 1994.

39. Grignon, D.J., Caplan, R., Sarkar, F.H., et al. P53 status and prognosis of locally advanced prostatic adenocarcinoma - a study based on RTOG 8610. J. Nat. Cancer Institute 89: 158-165, 1997.

40. Guillouf, C., Rosselli, F., Krishnaraju, K., Moustacchi, E., Hoffman, B. and Liebermann, D.A. p53 involvement in control of G_2 exit of the cell cycle: role in DNA damage-induced apoptosis. Oncogene 10: 2263-2270, 1995.

41. Hallahan, D.E., Virudachalam, S., Kuchibhotla, J., Kufe, D.W. and Weichselbaum, R.R. Membrane-derived second messenger regulates x-ray-mediated tumor necrosis factor alpha gene induction. Proc. Natl. Acad. Sci. U.S.A. 91: 4897-4901, 1994.

42. Halloran, P.J. and Fenton, R.G. Irreversible G_2/M arrest and cytoskeletal reorganization induced by cytotoxic nucleoside analogues. Cancer Research 58: 3855-3865, 1998.

43. Hanawalt, P.C. Role of transcription-coupled DNA repair in susceptibility to environmental carcinogenesis. Environ. Health Perspect. 104 Suppl 3: 547-551, 1996.

44. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75: 805-816, 1993.

45. Harris, C.C. p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology, and cancer risk assessment. Environ. Health Perspect. 104 Suppl 3: 435-439, 1996.

46. Iliakis, G. Cell cycle regulation in irradiated and nonirradiated cells. Semin.Oncol. 24:602-615, 1997.

47. Innocente, S.A., Abrahamson, J.L., Cogswell, J.P. and Lee, J.M. p53 regulates a G2 checkpoint through cyclin B1. Proc. Natl. Acad. Sci. U.S.A. 96:2147-2152, 1999.

48. Jayaraman, J. and Prives, C. Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell 81:1021-1029, 1995.

49. Kastan, M.B. Hot papers - oncology - participation of p53 protein in the cellular response to DNA damage by Kastan, M.B., Onyekwere, O., Sidransky, D., et al. Scientist. 7: 16 1993.

50. Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311, 1991.

51. Kastrinakis, W.V., Ramchurren, N., Rieger, K.M., et al. Increased incidence of p53 mutations is associated with hepatic metastasis in colorectal neoplastic progression. Oncogene 11: 647-652, 1995.

52. Kaufmann, W.K. and Kaufman, D.G. Cell cycle control, DNA repair and initiation of carcinogenesis. FASEB J. 7: 1188-1191, 1993.

53. Kolling, A., Maldonado, C., Ojeda, F. and Diehl, H.A. Membrane fluidity of microsomal and thymocyte membranes after X-ray and UV irradiation. Radiat. Environ. Biophys. 33: 303-313, 1994.

54. Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V. and Kastan, M.B. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl Acad. Sci. U.S.A. 89: 7491-7495, 1992.

55. Leach, S.D., Scatena, C.D., Keefer, C.J., et al. Negative regulation of wee1 expression and cdc2 phosphorylation during p53-mediated growth arrest and apoptosis. Cancer Research 58: 3231-3236, 1998.

56. Ling, C.C., Chen, C.H. and Fuks, Z. An equation for the dose response of radiation-induced apoptosis: possible incorporation with the LQ model. Radiother. Oncol. 33: 17-22, 1994.

57. Ling, C.C., Chen, C.H. and Li, W.X. Apoptosis induced at different dose rates: implication for the shoulder region of cell survival curves. Radiother. Oncol. 32: 129-136, 1994.

58. Lohrer, H.D. Regulation of the cell cycle following DNA damage in normal and Ataxia telangiectasia cells. Experientia 52: 316-328, 1996.

59. Lowe, S.W., Jacks, T., Housman, D.E. and Ruley, H.E. Abrogation of oncogene- associated apoptosis allows transformation of p53-deficient cells. Proc. Natl. Acad. Sci. U.S.A. 91: 2026-2030, 1994.

60. Lupulescu, A.P. Hormones, vitamins, and growth factors in cancer treatment and prevention - a critical appraisal [review]. Cancer 78: 2264-2280, 1996.

61. Macgrogan, D. and Bookstein, R. Tumour suppressor genes in prostate cancer. Semin. Cancer Biol. 8: 11-19, 1997.

62. Malcomson, R.D., Oren, M., Wyllie, A.H. and Harrison, D.J. p53-independent death and p53-induced protection against apoptosis in fibroblasts treated with chemotherapeutic drugs. Br. J. Cancer 72: 952-957, 1995.

63. McKenna, W.G., Bernhard, E.J., Markiewicz, D.A., Rudoltz, M.S., Maity, A. and Muschel, R.J. Regulation of radiation-induced apoptosis in oncogene-transfected fibroblasts: influence of H-*ras* on the G_2 delay. Oncogene 12: 237-245, 1996.

64. McKenna, W.G., Weiss, M.C., Bakanauskas, V.J., et al. The role of the H-*ras* oncogene in radiation resistance and metastasis. Int. J. Radiat. Oncol. Biol. Phys. 18: 849-859, 1990.

65. McKenna, W.G., Weiss, M.C., Endlich, B., et al. Synergistic effect of the v-*myc* oncogene with H-*ras* on radioresistance. Cancer Res. 50: 97-102, 1990.

66. Midgley, C.A., Owens, B., Briscoe, C.V., Thomas, D.B., Lane, D.P. and Hall, P.A. Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo. J. Cell Sci. 108: 1843-1848, 1995.

67. Moller, T. Skeletal metastases [review]. Acta Oncologica 35:Suppl 7: 125-136, 1996.

68. Nagasawa, H., Keng, P., Harley, R., Dahlberg, W. and Little, J.B. Relationship between gamma-ray-induced G_2/M delay and cellular radiosensitivity. Int. J. Radiat. Biol. 66: 373-379, 1994.

69. Oberosler, P., Hloch, P., Ramsperger, U. and Stahl, H. p53-catalyzed annealing of complementary single-stranded nucleic acids. EMBO J. 12: 2389-2396, 1993.

70. Park, M., Chae, H.D., Yun, J., et al. Constitutive activation of cyclin B1-associated cdc2 kinase overrides p53-mediated G_2/M arrest. Cancer Res. 60: 542-545, 2000.

71. Pellegata, N.S., Antoniono, R.J., Redpath, J.L. and Stanbridge, E.J. DNA damage and p53-mediated cell cycle arrest: a reevaluation. Proc. Natl. Acad. Sci. U.S.A. 93: 15209-15214, 1996.

72. Prasad, A.V., Mohan, N., Chandrasekar, B. and Meltz, M.L. Induction of transcription of "immediate early genes" by low-dose ionizing radiation. Radiat. Res. 143: 263-272, 1995.

73. Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M.E. and Tegtmeyer, P. The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. Proc. Natl. Acad. Sci. U.S.A. 92: 9455-9459, 1995.

74. Renzing, J. and Lane, D.P. p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. Oncogene 10: 1865-1868, 1995.

75. Rowan, Ludwig, R.L., Haupt, Y., et al. Specific loss of apoptotic but not cell-cycle arrest function in a human tumor derived p53 mutant. EMBO J. 15: 827-838, 1996.

76. Schwartz, D., Almog, N., Peled, A., Goldfinger, N. and Rotter, V. Role of wild type p53 in the G_2 phase - regulation of the gamma-irradiation-induced delay and DNA repair. Oncogene 15: 2597-2607, 1997.

77. Schwartz, D. and Rotter, V. p53-dependent cell cycle control: response to genotoxic stress. Semin.Cancer Biol. 8: 325-336, 1998.

78. Shiozaki, H., Doki, Y., Oka, H., et al. [E-cadherin expression and cancer invasion and metastasis]. Hum.Cell 6: 94-99, 1993.

79. Shiozaki, H., Oka, H., Inoue, M., Tamura, S. and Monden, M. E-cadherin mediated adhesion system in cancer cells. Cancer 77: Suppl S: 1605-1613, 1996.

80. Siegel, J., Fritsche, M., Mai, S., Brandner, G. and Hess, R.D. Enhanced p53 activity and accumulation in response to DNA damage upon DNA transfection. Oncogene 11: 1363-1370, 1995.

81. Symonds, H., Krall, L., Remington, L., et al. p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 78: 703-711, 1994.

82. Takasu, M., Tada, Y., Wang, J.O., Tagawa, M. and Takenaga, K. Resistance to apoptosis induced by microenvironmental stresses is correlated with metastatic potential in lewis lung carcinoma. Clin. Exp. Metastasis 17: 409-416, 1999.

83. Tamura, S., Shiozaki, H., Miyata, M., et al. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br. J. Surg. 83: 1608-1614, 1996.

84. Vikhanskaya, F., Vignati, S., Beccaglia, P., et al. Inactivation of p53 in a human ovarian cancer cell line increases the sensitivity to paclitaxel by inducing G_2/M arrest and apoptosis. Exp. Cell Res. 241: 96-101, 1998.

85. von Essen, C.F. Radiation enhancement of metastasis: a review. Clin. Exp. Metastasis 9: 77-104, 1991.

86. Wang, Y. and Iliakis, G. Prolonged inhibition by X-rays of DNA synthesis in cells obtained by transformation of primary rat embryo fibroblasts with oncogenes H-*ras* and v-*myc*. Cancer Res. 52: 508-514, 1992.

87. Wang, Y. and Prives, C. Increased and altered DNA binding of human p53 by S and G2/M but not G_1 cyclin-dependent kinases. Nature 376: 88-91, 1995.

88. Weichselbaum, R.R., Hallahan, D., Fuks, Z. and Kufe, D. Radiation induction of immediate early genes: effectors of the radiation-stress response. Int. J. Radiat. Oncol. Biol. Phys. 30: 229-234, 1994.

89. Williams, J.K. Principles of genetics and cancer. Semin. Oncol. Nurs. 13: 68-73, 1997.

90. Winters, Z.E., Ongkeko, W.M., Harris, A.L. and Norbury, C.J. P53 regulates cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G_2 arrest in human cells. Oncogene 17: 673-684, 1998.

CHAPTER II

THE INFLUENCE OF THE ONCOGENES *NRAS* AND *MYC* ON THE RADIATION SENSITIVITY OF CELLS OF A HUMAN MELANOMA CELL LINE

J. Pomp

I. J. M. Ouwerkerk

J. Hermans

J. Wondergem

C. J. Cornelisse

J. W. H. Leer

P. I. Schrier

Reprinted from Radiation Research 146: 374-381 1996
With permission from Radiation Research.

Abstract

Activation of certain oncogenes may alter the sensitivity of cells to ionising radiation. We studied the effect of oncogene activation on the radiation sensitivity of cells of a human melanoma cell line. The cell line IGR39D was transfected with the *MYC* oncogene, the proto-oncogene *NRAS*, *NRAS* activated by a point mutation (61-arginine), or a combination of mutated *NRAS* and *MYC*. Single-dose experiments showed a decreased survival after transfection with *MYC*, wild type *NRAS* or mutated *NRAS*. Cotransfection with *MYC* and mutated *NRAS* decreased survival up to 4 Gy, whereas at higher dose no shift in radiosensitivity was seen. Flow cytometry data indicated that differences in radiosensitivity could be explained at least in part by a difference in the distribution of cells in the phases of the cell cycle. After transfection of cells with either *NRAS* or *MYC*, the number of cells in G_1 phase decreased with a concomitant increase of cells in the G_2/M phase. When the cell line transfected with activated *NRAS* was manipulated so that the distribution of the cells in the phases of the cell cycle resembled that of the parental line at the time of radiation, the survival of the cells was improved. Similar experiments with the cell line containing *MYC* did not result in an alteration of the distribution of the cells in the cycle, or the survival after single dose fractions, suggesting the presence of a distinct mechanism for influencing radiation sensitivity. Both *NRAS* and *MYC* transfection decrease the radiation sensitivity of human melanoma cells, but the underlying mechanisms seem different. In conclusion, transfection with *NRAS* or *MYC* alone increases radiation sensitivity while transfection of cells containing *NRAS* with *MYC* restores resistance at higher doses.

Introduction

The role of oncogene activation in radiation response has been studied extensively. Most studies indicate an increased radioresistance after transfection with the oncogenes *Hras* and *HRAS*, [10,18,23-25,27,31,34,43] *Myc* and *MYC* [9,23], *ABL* [14] and *RAF* [9,19,30]. The majority of publications stating that oncogenes could increase radioresistance have dealt with rodent systems, whereas studies with cells of human cell lines, performed mostly on normal tissue, have not yielded consistent results. Alteration in radiation response in human cell lines containing a mutated *RAS* oncogene, consisted of either an improved survival [7,9,13,14,30], or a tendency for decreased survival [1]. According to several studies, mutated *RAS* did not seem to alter radiation response in human fibroblasts [35], human keratinocytes [26] or human retinoblasts [17]. Similar data were obtained for the *SRC* oncogene in hematopoietic progenitor cells [32].

To investigate the effect of oncogene activation on the radiosensitivity of tumour cells of human origin, we chose a melanoma tumour cell line. Human melanomas display relatively high resistance to radiotherapy. About 15% of melanomas had an activated *NRAS* oncogene (due to a mutation) [33,37] and 50% of a panel of tested melanoma cell lines has elevated expression of *MYC* [40]. For this purpose, cells of a human melanoma cell line without these oncogenes activated were transfected with the human *MYC* gene, commonly referred to as *c-myc*, *NRAS*, wild type or activated by a point mutation (61 arginine), or a combination of mutated *NRAS* and *MYC*. Clonogenic assays were used to measure survival after radiotherapy. Both the cell line transfected with *MYC* and the cell lines transfected with *NRAS* showed a decreased survival compared to the parental cell line, due in part to changes in distribution of cells in the phases of the cell cycle.

Material and Methods

Cell Culture and Transfection Procedures

The human melanoma cell line IGR39D, a subclone of IGR39 [3] and its transfectants were kept at 37°C in a 5% CO_2-flushed incubator in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin, 1.5% glutamine and 8% heat-inactivated fetal calf serum.

The IGR39D cell line and its *MYC* transfectant (IGRmyc) were described previously [33,40]. Briefly, an expression plasmid has been used, containing *MYC* exon 2 and 3 under the Moloney virus promotor as well as the neomycin phosphotransferase gene, which confers resistance to G418 [21]. Transfection with *MYC* was performed using the calcium phosphate method [16]. Transfection with either wild type genomic *NRAS* or mutated *NRAS* (61-arginine) was done by the same method. A plasmid containing the wild type genomic

NRAS or mutated *NRAS* (61-arginine), under the control of the CMV promotor, was transfected into IGR39D cells together with the neomycin phosphotransferase gene as a selection marker [39]. The cell lines used were the IGR-R1, containing a mutated *NRAS* (61-arginine), and IGR-Q2, IGR39D transfected with wild type *NRAS*. We renamed these cell lines IGRmtras and IGRwtras. To establish a cell line with both mutated *NRAS* and *MYC* oncogenes, IGRmtras cells were transfected with the *MYC* construct together with a plasmid containing guanosinephosphoribosyltransferase (gpt), as a selection marker. The resulting cell line was named IGRras/myc. To serve as a control, the human melanoma cell line IGR39D was transfected with a plasmid pCMVneo, containing the neomycin phosphotransferase gene only (IGRneo). The used cell lines are summarised in Table 1.

RNA isolation and Northern Blotting

Northern blotting was performed as described previously to reveal the presence of elevated *MYC* mRNA [28,40]. Briefly, cells were harvested by trypsinization and quick-frozen. Total RNA was isolated by the LiCl-urea method [4]. RNA (15 mg per lane) was fractionated by electrophoresis through a 1% agarose-formaldehyde gel and transferred onto nitrocellulose. Hybridization was performed sequentially with the following probes radiolabeled by the random primer method [12]: human *MYC*, a ClaI-EcoRI fragment of a human genomic clone spanning most of exon 3 and a rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA as a control for equal loading of RNA in each lane [41].

Protein Extraction and Western Analyses

For the detection of Myc protein, whole-cell protein extracts of cells of the melanoma cell line and its transfectants were prepared by high salt extraction [6,28]; 50 mg of protein was applied to SDS-PAGE and blotted onto nitrocellulose [8]. Immunoincubations were performed according to the protocol of the Western Light Kit (Tropix, Bedford MA). The

Table 1

Melanoma cell lines used in this study

IGR39D	Parental human melanoma cell line with no ras mutations and no elevated expression of *MYC*
IGRmyc	IGR39D transfected with exon 2 and 3 of the *MYC* oncogene
IGRmtras	IGR39D transfected with mutant 61-arg *NRAS*
IGRras/myc	IGRmtras supertransfected with *MYC* IGRwtras IGR39D transfected with wild type *NRAS*
IGRneo	IGR39D transfected with the neomycin phosphotransferase gene only

anti-c-myc mouse monoclonal antibody 9E10 (Cambridge Research Chemicals, Northwich, GB) was used at a dilution of 1:1000 [11]. Western analysis for the presence of mutated (61-arg) *NRAS* and the level of expression of wild type *NRAS* was performed as described previously [39]. Briefly, whole cell extracts were prepared using 0.14 M NaCl, 0.2 M triethanolamine, 0.2% Na-deoxycholate, and 0.5% Nonidet P-40, supplemented with 1mM phenylmethylsulphonyl fluoride and 0.2 mg/ml aprotonin. Extracts were quick-frozen twice and centrifuged to remove debris. Immunodetection was performed using the rat monoclonal antibody Y13-259 [15] at a dilution of 1:400.

For both the Myc and Nras protein, second antibody incubations were carried out, using goat anti-mouse or goat-anti-rat conjugated alkaline phosphatase (1:2500, Promega, Madison WI) and the chemiluminescent substrate CSPD™ [Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenylphosphate, Tropix] according to manufacturers' instructions. The chemiluminescent detection of the reaction product was accomplished by exposuríng the membranes to Fuji RX X-ray film for 0.5-15 minutes.

Plating Efficiency

Plating efficiencies for the different cell lines were assessed. Cells were plated in 60-mm dishes with 5 ml medium. As the plating efficiency decreased with increasing cell density, three different dilutions, each in triplicate, were plated per dose delivered. Plating efficiency was 40-50% for IGR39D, IGRneo, IGRmyc and IGRras/myc cells, 20% for IGRmtras and 15-20% for IGRwtras cells.

Irradiation

Cells from exponentially growing cultures were harvested by trypsinization and seeded in 60-mm dishes, 4 hours and 18 hours before a single dose was given. Cells taken from confluent growth were incubated 2 hours before treatment. Cells were irradiated at room temperature using a 5 or 6 MV accelerator at a dose rate of 4 Gy per minute. Dishes were placed on build-up material and irradiated with a PA field with the iso-center in the bottom of the dishes at cell level. Dose per fraction was 0-10 Gy. After radiation, dishes were returned to the incubator. At day 10-14, colonies were fixed with 96% ethanol and stained with Coomassie Brilliant Blue. Colonies with 50 or more cells were counted.

Flow Cytometry

The distribution of cells in the cycle at time of irradiation was evaluated by flow cytometry (FCM). Cells were trypsinized, resuspended and left to attach to the dish under the same conditions as cells used for radiation experiments. A total of $5x10^5$ cells per sample were harvested from a 90 mm dish and kept frozen in Vindelov's buffer [42].

Suspensions of isolated nuclei were prepared with the detergent-trypsin procedure of

Vindelov [38,42] using propidium iodide (PI) as a DNA stain. Red blood cells from trout, which have about 80% of the human diploid DNA content, were added to the samples as an internal standard. Analysis was performed on a FACScan (Becton Dickinson, Immunocytometry Systems, San Jose, CA) equipped with a 5 W argon laser. Histograms were generated from 10^4 events and displayed as linear fluorescence.

Cell Synchronization

To equalize the distribution of the cells in the cycle at the time of irradiation, two methods were used, aimed at reducing the G_2/M phase while increasing the G_1 phase. First, cells were plated 18 hours instead of 4 hours before irradiation. The extended plating time resulted in an increase in the number of IGRmtras cells in the G_1 phase concomitant with a reduction of the G_2/M phase. Second, cells were grown to confluence followed by 3 days in serum-depleted medium resulting, both for IGRmtras and IGRmyc, in an increased population of cells in G_1 phase, while the G_2/M phase was reduced.

Survival Curve

Each single-dose experiment was repeated three or four times. The mean survival per cell line was taken as the mean of the survival derived from three independent experiments. Survival curves were fitted according to the linear quadratic model using the following equation:

$^e\text{Log } S = - \alpha D - \beta D^2$,

where S = survival, D = radiation dose, and α (Gy^{-1}) and β (Gy^{-2}) are the fitted parameters [5]. The nonlinear regression module of NCSA (Number Cruncher Statistical Analysis) was used for the computations. The standard error was calculated from the survival of all experiments.

Results

Generation of cell lines

Cells of the human melanoma cell line IGR39D were transfected with the oncogenes *NRAS* and *MYC* in order to establish their role in radiation response. To evaluate whether a difference in radiation response is specifically related to a mutation of the *NRAS* oncogene, we also transfected the wild type *NRAS* in the IGR39D cell line and looked for differences in radiation sensitivity. The oncogene *Myc* is known to have a minor influence on radiosensitivity of rodent cell lines only, but the combination of the oncogenes *Myc* and *Hras* has been shown to have a synergistic effect on survival after irradiation, leading to a more radioresistant phenotype [25]. Therefore, we transfected the parental cell line as well as the

cell line that contained mutated *NRAS* (IGRmtras) with *MYC*. IGR39D cells transfected with the neomycin phosphotransferase gene served as a control.

RNA isolation and Northern blotting were performed to establish the presence of *MYC* mRNA in the various transfected cell lines. Low endogenous *MYC* expression was seen in the parental cell line IGR39D and in the cell lines IGRmtras and IGRwtras (Figure 1A, band marked with star). The cell lines IGRmyc and IGRras/myc showed high levels of transfected *MYC* mRNA as concluded from the increase in expression of the marked band as well as a second (longer) transcript transcribed from the transfected construct (Fig. 1A)

Western analysis was performed to examine the presence of Myc protein. High levels of Myc protein were found in the cell lines IGRmyc and IGR ras/myc (Figure 1B). IGRras/myc cells have a lower level then IGRmyc; the latter cell line was derived by transfecting IGRmtras with *MYC*. Much lower levels of endogenous Myc protein were detected in the parental cell line, IGR39D, in the *NRAS*-transfected cell lines IGRmtras and IGRwtras, and in IGRneo cells (Figure 1B).

The levels of Ras protein, wild type $p21^{N\text{-}raswt}$, and mutated $p21^{N\text{-}ras\ 61ARG}$, were also assayed by western analysis. The wild-type Ras protein consists of two bands, an unmodified and a farnesylated from, as indicated in Fig. 1C. The mutated protein is represented by one band with higher mobility. IGR39D, IGRmyc and IGRneo cells show only the wild-type bands (Fig. 1C). In contrast, IGRmtras and IGRras/myc cells clearly show expression of the mutated $p21^{N\text{-}ras\ 61arg}$. The cell line transfected with wild type *NRAS* shows elevated expression of $p21^{wtras}$ compared with the other cell lines.

The doubling time of IGR39D was 26 hours. IGRneo and IGRmyc showed a reduced doubling time (20 respectively 12.5 hours) and transfection with wild type ras lead to increased doubling time (33 hours). Transfection with mutated *NRAS* did not affect the doubling time (27 hours).

Survival

The radiosensitivity of the cell lines described above was assessed by colony-forming assays as described in Materials and Methods. In Figure 2A the influence of transfection with the *MYC* oncogene on survival after irradiation is visualised. The IGRmyc cell line shows an increased radiosensitivity compared to the parental cell line. Transfection with the neomycin phosphotransferase plasmid pCMVneo, containing the neomycin phosphotransferase gene only, however, did not influence the survival.

In Figure 2B the influence of transfection with either wild-type *NRAS* (IGRwtras) or mutated *NRAS* on radiation survival is shown. Both wild type *NRAS* and mutated *NRAS* lowered the survival. When the IGR39D cell line was transfected with mutated *NRAS* as well as *MYC*, the survival decreased in the dose range from 0-4 Gy (Figure 2C).

The radiobiological parameters α (Gy^{-1}) and β (Gy^{-2}), obtained after fitting the data according to the linear quadratic model, as well as the 2 Gy survival (SF_2) are listed in Table

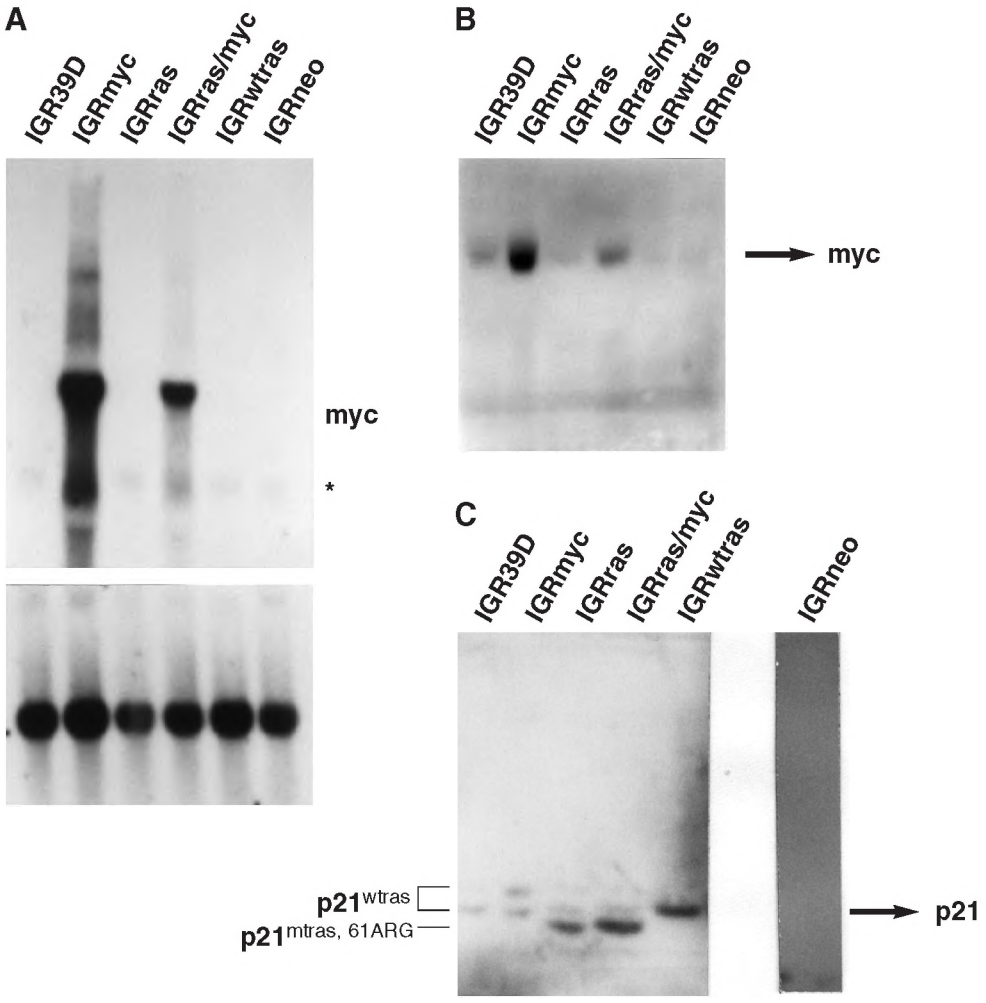


Figure 1

Expression of MYC and NRAS in transfected melanoma cell lines.

A: Northern blotting of cell lines as indicated.

B,C: Western blotting of cell lines as indicated. Antibodies against MYC (B) and $p21^{N\text{-}ras}$ (C), were used.

II. Transfection with mutated *NRAS*, wild type *NRAS* and *MYC* increased the α-component leading to a reduced shoulder of the survival curve. The effect was most pronounced in IGRmtras cells. Transfection of the IGRmtras cell line with *MYC* (IGRras/myc) also showed an increased α component. The increase in α was accompanied by a reduced SF_2 (Table 2). Transfection with mutated *NRAS*, (cell lines IGRmtras and IGRras/myc) but not wild type *NRAS* or *MYC*, also decreased the β-component of the survival curve, suggesting a

Table 2.
Radiobiological parameters for cells plated 4 hours before irradiation

Cell line	α (SE)[a]	ß (SE)	α/ß	SF_2[b] (SE)
IGR39D	0.22 (2.10^{-2})	0.030 (3.10^{-3})	7.3	0.63 (0.061)
IGRmyc	0.36 ($2.5.10^{-2}$)	0.030 (3.10^{-3})	12.0	0.48 (0.056)
IGRmtras	0.46 ($2.5.10^{-2}$)	0.018 (3.10^{-3})	25.5	0.35 (0.052)
IGRras/myc	0.38 ($2.4.10^{-2}$)	0.015 (3.10^{-3})	25.3	0.40 (0.093)
IGRwtras	0.34 ($3.4.10^{-2}$)	0.045 (4.10^{-3})	7.5	0.37 (0.064)
IGRneo	0.17 ($2.2.10^{-2}$)	0.040 (3.10^{-3})	4.25	0.62 (0.066)

[a] One standard error (SE) of the mean is given

[b] 2 Gy survival fraction derived from radiobiological parameters

decreased capacity to repair sublethal damage. Wild-type *NRAS* increased the β-component. When calculating the α/β ratio it is seen that transfection with the mutated *NRAS* oncogene (IGRmtras and IGRmyc/ras) lead to a rise in a/b ratio compared to the parental line. The parameters α and β as well the SF_2 for the IGRneo resemble the parental cell line IGR39D.

Distribution of Cells in the Cell Cycle

To investigate whether the increased radiosensitivity after *MYC* or *NRAS* transfection is related to the phase of the cell cycle, we tested whether any changes in he distribution of the cells in the cycle had occurred due to transfection with the oncogenes. Cells used for flow cytometry underwent the same treatment as cells used for radiation experiments: trypsinization from cultures in 70% confluence and incubation for 4 hours. The results are presented in Table III, experimental setting A. A change in the distribution of the cells in the cycle did occur after transfection with either *NRAS* (mutated or wild type) or *MYC*. Compared to the parental cell line IGR39D, the G_1 phase was reduced, whereas the fraction of cells in G_2/M phase was increased. The cells transfected with either *NRAS*, *MYC* or both showed a slight increase in the S phase fraction.

Survival after Cell Synchronization

To test whether the difference in distribution of cells in the cycle could fully explain the altered radiosensitivity, cells were irradiated in the same distribution of cells in the cycle as the parental cell line. Earlier experiments had demonstrated, that extending plating time increased the G_1 phase and decreased the G_2/M phase of the cell line transfected with mutated *NRAS*. Therefore, we performed irradiation experiments with a plating time of 18 hours instead of 4 hours before treatment. No change in cell cycle distribution of either IGR39D,

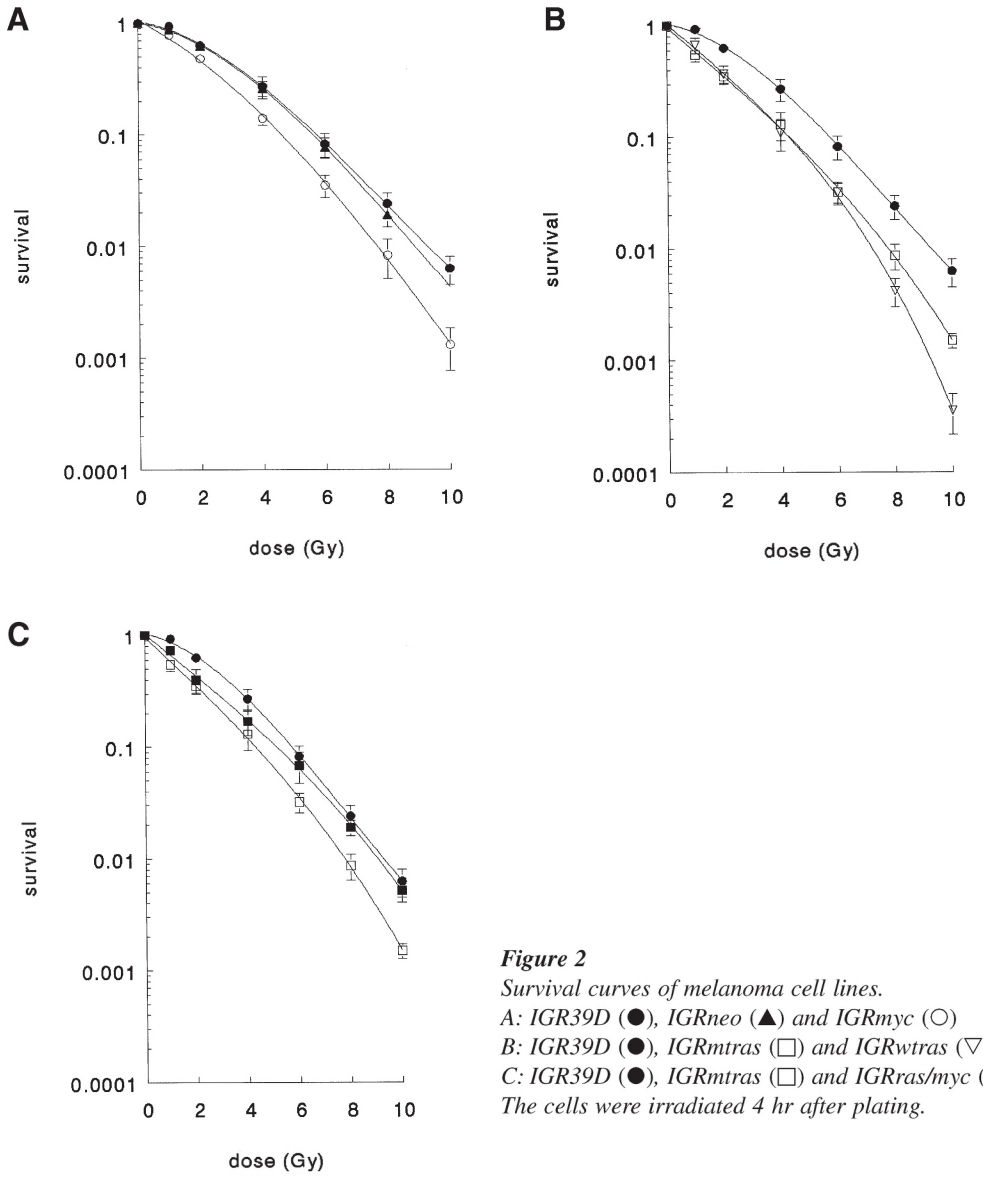


Figure 2
Survival curves of melanoma cell lines.
A: IGR39D (●), IGRneo (▲) and IGRmyc (○)
B: IGR39D (●), IGRmtras (□) and IGRwtras (▽)
C: IGR39D (●), IGRmtras (□) and IGRras/myc (■)
The cells were irradiated 4 hr after plating.

or IGRneo cells in the cycle occurred, while IGRmyc cells showed a small reduction in G_2/M phase (Table III, experimental setting B). Survival of either cell line was not affected (Figure 3A, 3B and 3D). The IGRmtras cell line, however, showed a more pronounced alteration in distribution of cells in the cycle (Table III, experimental setting B), with an increase in G_1 phase and reduction in G_2/M, concomitant with an increased SF_2. In this case, survival was increased (Figure 3C)

Table 3.

Experimental Setting A[(1)]

	G1 (%)	*S (%)*	*G2/M (%)*	*SF2*	*(SE)*[a]
IGR39D	68	22	10	0.63	(0.061)
IGRmyc	42	30	28	0.48	(0.056)
IGRmtras	45	27	28	0.35	(0.052)
IGRras/myc	49	27	24	0.40	(0.093)
IGR/wtras	57	25	18	0.37	(0.064)
IGR/neo	57	31	12	0.61	(0.066)

Experimental Setting B

	G1 (%)	*S (%)*	*G2/M (%)*	*SF2*	*(SE)*[a]
IGR39D	69	24	7	0.63	(0.078)
IGRmyc	40	40	20	0.48	(0.062)
IGRmtras	62	27	11	0.54	(0.067)
IGRras/myc	nd[b]	nd	nd	nd	
IGR/wtras	nd	nd	nd	nd	
IGR/neo	60	32	8	0.57	(0.10)

Experimental Setting C

	G1 (%)	*S (%)*	*G2/M (%)*	*SF2*	*(SE)*[a]
IGR39D	66	27	7	0.56	(0.098)
IGRmyc	67	21	12	0.43	(0.049)
IGRmtras	66	25	9	0.44	(0.071)
IGRras/myc	nd	nd	nd	nd	
IGR/wtras	nd	nd	nd	nd	
IGR/neo	67	28	5	0.57	(0.098)

(1) Experimental settings:
A: % G_1 when cells are plated 4 hours before irradiation
B: % S when cells are plated 18 hours before irradiation
C: % G_2/M when cells are plated from confluent growth in serum depleted medium, 2 hours before irradiation

[a] One standard error (SE) of the mean is given

[b] nd: not determined

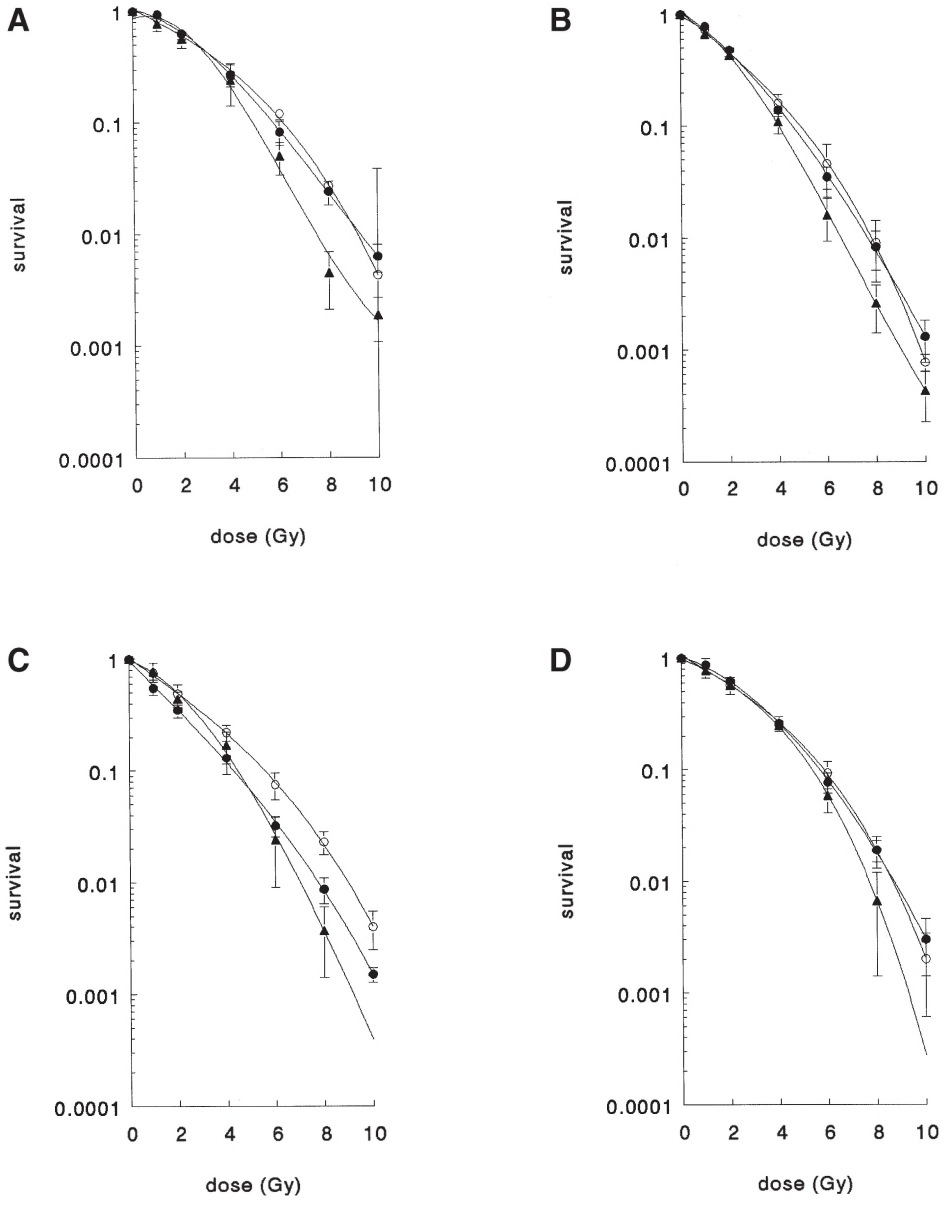


Figure 3

Survival curves of melanoma cell lines.

A: IGR39D plated 4 hours (●), 18 hours (○) and taken from confluent growth (▲) before irradiation.

B: IGRmyc plated 4 hours (●), 18 hours (○) and taken from confluent growth (▲) before irradiation.

C: IGRmtras plated 4 hours (●), 18 hours (○) and taken from confluent growth (▲) before irradiation.

D: IGRneo plated 4 hours (●), 18 hours (○) and taken from confluent growth (▲) before irradiation.

The second method we used to force cell lines in the same distribution of cells in the cycle was serum depletion of confluent cultures for three days. Cells were trypsinized and seeded two hours before irradiation to prevent them from entering the cell cycle before treatment. The distribution of cells in parental cell line IGR39D and IGRneo was not altered by this manipulation (Table III, experimental setting C) nor was there a change at low dose, but overall survival was decreased (Figure 3A, 3D). The G_1 phase of the IGRmtras cell line increased and there was a small gain in survival at the initial part of the curve (up to 4 Gy) whereas survival after higher doses was comparable to the outcome of the 4-h experiments (figure 3C). The SF_2 was slightly higher than from the 4-h experiments (0.44 vs. 0.35). IGRmyc with an increase in G_1 phase (Table III, experimental setting C), had no increase in SF_2 (Figure 3B). The combined experiments clearly show that prolonged plating time results in an increase in G_1 phase, together with a decrease in G_2/M phase and an increased survival of IGRmtras cells. Increasing the G_1 phase while reducing G_2/M by taking cells from confluent growth, however, does not lead to an expected increase in survival in the case of IGRmtras cells. IGRmyc cells had no clear shift in cell cycle distribution and SF_2 remained unaltered after 18 hours incubation before treatment. When IGRmyc cells were treated after growth in serum depleted medium, no gain in survival was seen although G_2/M phase decreased while G_1 phase increased.

Discussion

Role of oncogenes

There is increasing interest about the role of oncogenes in the malignant transformation of normal cells in relation to their impact on treatment modalities. The influence of oncogenes on survival after irradiation has attracted the attention of many radiation oncologists. The reports concerning an altered radiation response with oncogene expression appeared in the late 1980s [20,22,29,34]. These publications dealt mostly with rodent systems and indicated an increased radioresistance after transfection of cell lines with the oncogenes *Myc, Hras or Raf*. Further examination of the literature for other cell types such as normal human cells, revealed no clear picture of the role of oncogenes in radiosensitivity. The data of the rodent cell lines showed consistent results, and a number of explanations for the effect of oncogene activation on radiation response was put forward. First, the increased radioresistance of rodent cells transfected with the oncogenes *Hras* and *Myc* could be attributed to an increased G_2-phase delay [23,36]. The G_2- phase delay occurs after DNA damage to allow for repair before enter mitosis and is dependent on dose. Second, the distribution of cells in the cycle could play a role. Radiosensitivity is different for the different phases of the cell cycle, the G_2/M phase being the most sensitive and the S phase the most resistant. Also, a difference in radiation response between cell lines was found to be more pronounced in the

late S phase than in other phases of the cell cycle [10]. Third, primary rat embryo fibroblasts transfected with *Hras* and *Myc*, besides showing an increased G_2/M-phase delay, also showed prolonged inhibition of DNA synthesis resulting in a radioresistant phenotype [43].

Effects of NRAS and MYC

Human melanoma cell lines in general are known for their radioresistance in the clinic. A number of activated oncogenes, including *NRAS* and *MYC*, have been found in a considerable percentage of melanomas. To see whether this intrinsic radioresistance might be dependent on activation of these oncogenes, cells of a human melanoma cell line (IGR39D) were transfected with the *MYC* and *NRAS* oncogenes. We did find a different radiosensitivity after transfection with *NRAS* or *MYC* into the human melanoma cell line IGR39D. After transfection with the *NRAS* wild-type gene, with *NRAS* activated by a pointmutation or with *MYC*, the radiosensitivity was increased. IGRras/myc, containing both mutated *NRAS* and *MYC*, only showed reduced survival up till a dose of 4 Gy. To investigate whether a difference in distribution of cells in the cycle coincided with the altered radiosensitivity, analysis by flow cytometry was performed. Data for the parental cell line and the daughter cell lines transfected with mutated *NRAS*, *MYC* or both, indicated a shift in cell cycle distribution: Together with a decrease in the G_1 phase there was an increase in the G_2/M phase. In one particular case (IGRmtras), we were able to partially reverse the effect of activated *NRAS*; i.e., increased radiosensitivity was reversed by extending the plating time before irradiation. This effect is not due to a relatively increased cell number since the doubling time of IGRmtras was higher than that of IGRneo and IGRmyc cells. Flow cytometry showed that the G_2/M phase had decreased while G_1 phase had increased, resembling the distribution of the cells in the parental cell line in the cell cycle. This indicates that, at least in the case of IGRmtras cell line, the increased G_1 phase correlates with higher radioresistance. This phenomenon does not hold for the other cell lines and is apparently associated only with IGRmtras.

Conditions Critical for Radiosensitivity

Starvation of the cell lines IGR39D, IGRmyc and IGRneo at confluence increased their radiosensitivity to doses above 4 Gy. This effect was less pronounced in IGRmtras cells. The treatment apparently changes processes in the cell that determine the sensitivity for ionising radiation. One explanation may be an increased sensitivity for radiation-induced apoptosis. This could be caused by activation of *MYC* in the serum-depleted cultures after reconstitution with complete medium. Activation of *MYC* is related to an increase in radiation-induced apoptosis and as activation of *NRAS* can inhibit radiation-induced apoptosis [2] this may explain why the survival of IGRmtras was not as strongly reduced as the other cell lines.

Finally, our data show that the time point of irradiation is of extreme importance for proper evaluation of the effect of *NRAS*, because extension of the plating time would part-

ly have masked the effect of the oncogene (*cf* Fig. 3c). This stresses that experimental conditions are crucial for the detection of effects of agents that influence radiosensitivity.

Conclusions

Although in rodent systems the presence of activated oncogenes, *Hras* and *myc*, is repeatedly associated with a radioresistant phenotype, this was not consistently observed in human cell lines. Our present study shows that this could not be observed for human melanoma cell lines, known for their radioresistance and a relatively high percentage of *MYC* and *NRAS* activation. We rather found an increased radiosensitivity, whereas supertransfection of cells tranfected with *NRAS* restores the radioresistance, at least at higher doses. Most probably, *MYC* and *NRAS* affect radiosensitivity by different mechanisms, which in combination may interfere with each other, leading to a reduced effect of cells transfected with *MYC* as well as *NRAS*.

Acknowledgements

The authors wish to thank C. Hoogstraten for expert technical assistance and A. Boon and the technical staff of the Department of Clinical Oncology for their help with the radiation protocol. We thank N.J. Kuipers-Dijkshoorn for the provision and help with the DNA flow cytometry.

Literature

1. Alapetite, C., Baroche, C., Remvikos, Y., Goubin, G. and Moustacchi, E. Studies on the influence of the presence of an activated ras oncogene on the in vitro radiosensitivity of human mammary epithelial cells. Int. J. Radiat. Biol. 59: 385-396, 1991.

2. Arends, M.J., McGregor, A.H., Toft, N.J., Brown, E.J. and Wyllie, A.H. Susceptibility to apoptosis is differentially regulated by c-myc and mutated Ha-ras oncogenes and is associated with endonuclease availability. Br. J. Cancer 68: 1127-1133, 1993.

3. Aubert, C., Rouge, F. and Galindo, J.R. Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation in vitro. J. Nat. Cancer Inst. 64: 1029-1040, 1980.

4. Auffray, C. and Rougeon, F. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. 107: 303-314, 1980.

5. Barendsen, G.W. Dose fractionation, dose rate, and isoeffect relationships for normal tissue response. Int. J. Radiat. Oncol. Biol.Phys. 8: 1981-1997, 1982.

6. Bernards, R. N-myc disrupts protein kinase C-mediated signal transduction in neuroblastoma. EMBO J. 10: 1119-1125, 1991.

7. Bruyneel, E.A., Storme, G.A., Schallier, D.C.C., Vandenberge, D.L., Hilgard, P. and Mareel, M.M. Evidence for abrogation of oncogene-induced radioresistance of mammary cancer cells by hexadecylphosphocholine in vitro. Eur. J. Cancer 29A: 1958-1963, 1993.

8. Burnette, W.N. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195-203, 1981.

9. Chang, E.H., Pirollo, K.F., Zou, Z.Q., et al. Oncogenes in radioresistant, noncancerous skin fibroblasts from a cancer-prone family. Science 237: 1036-1039, 1987.

10. Cheong, N., Wang, Y. and Iliakis, G. Radioresistance induced in rat embryo cells by transfection with the oncogenes H-ras plus v-myc is cell cycle dependent and maximal during S and G2. Int. J. Radiat. Biol. 63: 623-629, 1993.

11. Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol.Cell Biol. 5: 3610-3616, 1985.

12. Feinberg, A.P. and Vogelstein, B. A technique for radiolabeling DNA restriction

endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-13, 1983.

13. FitzGerald, T.J., Henault, S., Sakakeeny, M., et al. Expression of transfected recombinant oncogenes increases radiation resistance of clonal hematopoietic and fibroblast cell lines selectively at clinical low dose rate. Radiat. Res. 122: 44-52, 1990.

14. FitzGerald, T.J., Santucci, M.A., Das, I., Kase, K., Pierce, J.H. and Greenberger, J.S. The V-ABL, C-FMS, or V-MYC Oncogene Induces Gamma- Radiation Resistance of Hematopoietic Progenitor Cell Line 32d CL 3 at Clinical Low Dose Rate. Int. J. Radiat. Oncol. Biol. Phys. 21: 1203-1210, 1991.

15. Furth, M.E., Davis, L.J., Fleurdelys, B. and Scolnick, E.M. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. J.Virol. 43: 294-304, 1982.

16. Graham, F.L. and Eb, A.J. Transformation of rat cells by DNA of human adenovirus 5. Virology 54: 536-539, 1973.

17. Grant, M.L., Bruton, R.K., Byrd, P.J., et al. Sensitivity to ionising radiation of transformed human cells containing mutant ras genes. Oncogene 5: 1159-1164, 1990.

18. Hermens, A.F. and Bentvelzen, P.A. Influence of the H-ras oncogene on radiation responses of a rat rhabdomyosarcoma cell line. Cancer Res. 52: 3073-3082, 1992.

19. Kasid, U., Pfeifer, A., Weichselbaum, R.R., Dritschilo, A. and Mark, G.E. The raf oncogene is associated with a radiation-resistant human laryngeal cancer. Science 237: 1039-1041, 1987.

20. Kasid, U.N., Weichselbaum, R.R., Brennan, T., Mark, G.E. and Dritschilo, A. Sensitivities of NIH/3T3-derived clonal cell lines to ionizing radiation: significance for gene transfer studies. Cancer Res. 49: 3396-3400, 1989.

21. Lau, Y.F. and Kan, Y.W. Versatile cosmid vectors for the isolation, expression, and rescue of gene sequences: studies with the human alpha-globin gene cluster. Proc. Natl. Acad. Sci. U.S.A. 80: 5225-5229, 1983.

22. Ling, C.C. and Endlich, B. Radioresistance induced by oncogenic transformation. Radiat. Res. 120: 267-279, 1989.

23. McKenna, W.G., Iliakis, G., Weiss, M.C., Bernhard, E.J. and Muschel, R.J. Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc. Radiat. Res. 125: 283-287, 1991.

24. McKenna, W.G., Weiss, M.C., Bakanauskas, V.J., et al. The role of the H-ras oncogene in radiation resistance and metastasis. Int. J. Radiat. Oncol. Biol.Phys. 8: 849-859,

1990.

25. McKenna, W.G., Weiss, M.C., Endlich, B., et al. Synergistic effect of the v-myc oncogene with H-ras on radioresistance. Cancer Res. 50: 97-102, 1990.

26. Mendonca, M.S., Boukamp, P., Stanbridge, E.J. and Redpath, J.L. The radiosensitivity of human keratinocytes: influence of activated c-H-ras oncogene expression and tumorigenicity. Int. J. Radiat. Biol. 59: 1195-1206, 1991.

27. Ong, A., Li, W.X. and Ling, C.C. Low-dose-rate irradiation of rat embryo cells containing the h-ras oncogene. Radiat. Res.134: 251-255, 1993.

28. Peltenburg, L.T.C., Steegenga, W.T., Krüse, K.M. and Schrier, P.I. C-myc-induced Natural Killer cell sensitivity of human melanoma cells is reversed by HLA-B27 transfection. Eur. J. Immunol. 22: 2737-2740, 1992.

29. Pirollo, K.F., Garner, R., Yuan, S.Y., Li, L., Blattner, W.A. and Chang, E.H. Raf involvement in the simultaneous genetic transfer of the radioresistant and transforming phenotypes. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 55: 783-796, 1989.

30. Pirollo, K.F., Tong, Y.A., Villegas, Z., Chen, Y. and Chang, E.H. Oncogene-transformed NIH-3T3 cells display radiation resistance levels indicative of a signal transduction pathway leading to the radiation-resistant phenotype. Radiat. Res. 135: 234-243, 1993.

31. Samid, D., Miller, A.C., Rimoldi, D., Gafner, J. and Clark, E.P. Increased radiation resistance in transformed and nontransformed cells with elevated ras proto-oncogene expression. Radiat. Res. 126: 244-250, 1991.

32. Santucci, M.A., Anklesaria, P., Anderson, S.M., et al. The v-src oncogene may not be responsible for the increased radioresistance of hematopoietic progenitor cells expressing v-src. Radiat. Res. 129: 297-303, 1992.

33. Schrier, P.I., Versteeg, R., Peltenburg, L.T.C., Plomp, A.C., Van 't Veer, L.J. and Krüse-Wolters, K.M. Sensitivity of melanoma cell lines to natural killer cells: a role for oncogene-modulated HLA expression? Semin. Cancer Biol. 2: 73-83, 1991.

34. Sklar, M.D. The ras oncogenes increase the intrinsic resistance of NIH 3T3 cells to ionizing radiation. Science 239: 645-647, 1988.

35. Su, L.N. and Little, J.B. Transformation and radiosensitivity of human diploid skin fibroblasts transfected with activated ras oncogene and SV40 T-antigen. Int. J. Radiat. Biol. 62: 201-210, 1992.

36. Su, L.N. and Little, J.B. Prolonged cell cycle delay in radioresistant human cell lines transfected with activated ras oncogene and/or simian virus 40 T-antigen. Radiat. Res

133: 73-79, 1993.

37. Van 't Veer, L.J., Burgering, B.M.T., Versteeg, R., et al. NRAS mutations in human cutaneous melanoma on sun-exposed body sites. Mol.Cell. Biol. 9: 3114-3116, 1989.

38. Van den Ingh, H.F., Griffioen, G. and Cornelisse, C.J. Flow cytometric detection of aneuploidy in colorectal adenomas. Cancer Res. 45: 3392-3397, 1985.

39. Van Elsas, A., Van Deursen, E., Wielders, R., van den Berg Bakker, C.A. and Schrier, P.I. RAS oncogene activation does not induce sensitivity to natural killer cell-mediated lysis in human melanoma. J. Invest. Dermatol. 103: 117S-121S, 1994.

40. Versteeg, R., Noordermeer, I.A., Krüse-Wolters, K.M., Ruiter, D.J. and Schrier, P.I. C-myc downregulates class I HLA expression in human melanomas. EMBO J. 7: 1023-1029, 1988.

41. Versteeg, R., Peltenburg, L.T.C., Plomp, A.C. and Schrier, P.I. High expression of the c-myc oncogene renders melanoma cells prone to lysis by natural killer cells. J. Immunol. 143: 4331-4337, 1989.

42. Vindelov, L.L., Christensen, I.J. and Nissen, N.I. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3: 323-327, 1983.

43. Wang, Y. and Iliakis, G. Prolonged inhibition by X-rays of DNA synthesis in cells obtained by transformation of primary rat embryo fibroblasts with oncogenes H-ras and v-myc. Cancer Res. 52: 508-514, 1992.

CHAPTER III

THE INFLUENCE OF THE ONCOGENE ***NRAS*** ON CELL CYCLE DELAY IN A HUMAN MELANOMA CELL LINE WITH REDUCED RADIORESISTANCE.

J. Pomp

I.J.M. Ouwerkerk

C. Hoogstraten

J. Wondergem

J. Davelaar

J.W.H. Leer

P.I. Schrier

Reproduced from: Oncology Reports 7: 663-667, 2000
With permission from Professor D.A. Spandidos

Abstract

Background and purpose: In a previous study we found that transfection of a human melanoma cell line with the oncogene *NRAS* led to increased radiosensitivity as measured by clonogenic assays. Since a shift in radiosensitivity is often correlated with altered G_2/M delay, we investigated whether this was also the case in this oncogene containing melanoma cell line (IGRras). *Material and methods*: A human melanoma cell line, stably transfected with mutated *NRAS*, and its parental cell line transfected with the neomycin phosphotransferase gene only (IGRneo), were irradiated with 5 Gy and cell cycle distribution was measured at hourly time intervals by DNA staining with propidium iodide. Next, the effect of ionising radiation on the duration of the S phase was determined by pulse labelling cells with BrdUrd before irradiation. *Results*: Both cell lines showed a radiation induced G_2/M delay, which was most prolonged for the *NRAS* transfected cell line. After 5 Gy, the S phase duration was unaltered, although the shape of the relative movement (RM) curves was slightly different. No G_1 delay was observed in either cell line. *Conclusions*: *NRAS* transfection in a melanoma cell line leads to prolonged G_2/M delay after radiotherapy. This prolongation is associated with increased radiosensitivity and not with radioresistance. These data throw doubt on the use of oncogene expression or G_2/M delay as predictors of radiosensitivity.

Introduction

Oncogenes that are responsible for the malignant transformation and immortalization of cultured cells like *Hras* and *Myc* may also play a role in the response to ionising radiation. In literature the original work on this subject dealt with mostly rodent cell lines. A large amount of data has shown that when expressed in these cell lines the oncogenes *Hras* and *Myc* are associated with increased radioresistance [11,13,19,30]. However, some reports using other cell lines [1,21,24], have not confirmed this phenomenon. Furthermore the rodent data showed that this increased radioresistance was accompanied by an increased G_2/M delay [18] which has also been reported for normal human diploid cell lines [32], suggesting a correlation between the length of the G_2/M delay and intrinsic radiosensitivity. This phenomenon could be a mechanistic explanation for a radioresistant phenotype since more time can be dedicated to repair the radiation induced DNA damage before the cell enters mitosis.

To further explore the relevance of this phenomenon and its impact on differences in radiosensitivity with regard to human tumours, a melanoma cell line was transfected with mutated *NRAS* and its impact on radiosensitivity was studied. Human melanomas are generally known as radioresistant tumours. The nature of the intrinsic radioresistance of melanomas remains unsolved up till now. However, it is known that a large percentage of these melanomas overexpress oncogenes; about 50% of human melanoma cell lines have elevated expression of *MYC* [35] and about 15% contain mutations in the *NRAS* oncogene [26,33].

Previous work of our laboratory on a human melanoma cell line did not reveal increased radioresistance after oncogene transfection: On the contrary the *NRAS* containing cell line was more radiosensitive [24]. The next step was to explore the hypothesis that this reduced survival after radiation would be accompanied by a shorter G_2/M delay in the melanoma cell line that was transfected with the *NRAS* oncogene. The aim of the study was to measure cell cycle effects after radiation in a parental cell line which contained the neomycin phosphotransferase gene as a selection marker only and an *NRAS* transfected cell line. We examined how cell cycle prolongation correlated with altered radiosensitivity. The results and possible mechanisms are discussed in the scope of the present literature.

Material and Methods

Cell lines

The human melanoma cell line IGR39D, a subclone of IGR39 [2] was transfected with mutated *NRAS* (61-arginine) [34]. The cell line taken from this study, IGR-R1, was

renamed IGRras. Mutated *NRAS* expression was confirmed by Northern blotting for mRNA and western blotting for protein [24,27,34]. The neomycin phosphotransferase gene was co-transfected as a selection marker. The cell line that contains the neomycin phosphotransferase gene only served as a control (IGRneo). All cells were kept at 37°C in a 5% CO_2 flushed incubator in Dulbecco's Minimal Essential Medium (DMEM), supplemented with penicillin/streptomycin, 2.8 mM glutamine and 8% heat inactivated foetal calf serum. The SF_2 as measured by clonogenic assays is 0.62 ± 0.06 for the parental cell line (IGRneo) and 0.35 ± 0.05 for the RAS transfected cell line (IGRras) [24].

Cell cycle alterations after radiotherapy.

To examine the shift in cell cycle distribution at different time intervals after irradiation, propidium iodide (PI) staining was used. Cells were irradiated with a 6 MV accelerator with a dose of 5 or 10 Gy. For S phase progression, cells were pulse-labelled for 5 minutes by adding BrdUrd (bromodeoxyuridine) at a final concentration of 1 mM to the dishes. After washing and replacing them in BrdUrd-free medium, the cells were exposed to 5 Gy, delivered with 250 kV at a dose rate of 0.75 Gy per minute, at ambient temperature. Cells in exponential growth were used. Dishes were returned to the incubator and harvested every hour. Cells that had incorporated BrdUrd were stained with anti-BrdUrd (a mouse antibody from Partec, Switzerland) and visualised using a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma F-0257). Total DNA content was counterstained with PI and cell cycle analysis was performed on a FACScan (Becton Dickinson, San José, CA) using the Lysis II programme.

By labelling cells with BrdUrd, the fraction of cells in the S phase as a function of time post-labelling can be measured and their sequential transition through the S phase. This makes it possible to measure the duration of the S phase both for untreated and irradiated cells. The total duration of the cell cycle was also derived from these data. The relative movement (RM) is a measure for the transition of cells through the S phase. The RM is defined to be zero for cells in G_1 and 1.0 for cells in the G_2/M phase. The duration of the S phase was calculated according to the method as proposed by Begg et al. by extrapolation to RM equals unity, when all cells have reached G_2 or beyond [6]. The relative movement was determined at different time intervals for irradiated and un-irradiated cultures, and used to determine the length of the S phase.

Results

Cell cycle alterations after radiotherapy

Figure 1 shows cell cycle alterations as a result of 5 Gy irradiation with the 6 MV machine. The P.I. data of the experiments performed with the 250 KV were consistent with

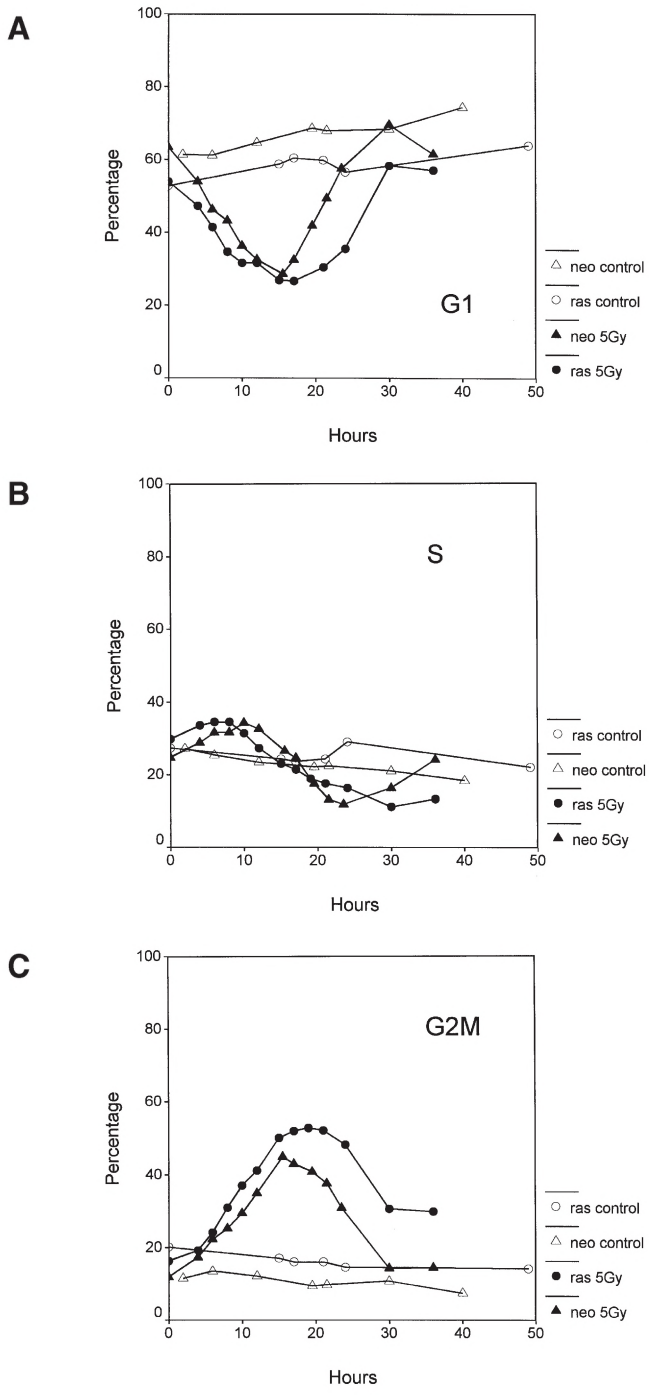


Figure 1

Cell cycle distribution after irradiation with 5 Gy for IGRneo and IGRras (A, G_1 phase; B, S-phase; C, G_2/M phase): ▲, (neo, 5 Gy); ●, (ras 5 Gy); △, (neo control); ○, (ras control).

the accelerator data and therefore not shown separately. In figure 1a it is seen that both cell lines have a decrease in G_1 phase; no accumulation in G_1 occurs. Both cell lines reach their nadir at the same time (12 hours). The IGRras showed a longer nadir (12-18 hours) and returned back to normal slower than the IGRneo cell line (27 hours vs. 22 hours).

The S phase of both cell lines shows a small and parallel increase immediately after treatment with a significant drop after 12 hours (Figure 1b). This time point of 12 hours post irradiation coincides with the nadir of the G_1 phase. At the end of the observation period the S phase of IGRras is not back to normal while the S phase of IGRneo is.

Both cell lines showed a G_2/M delay, reflected by an accumulation in G_2/M, as expected after radiotherapy (Figure 1c). This G_2/M delay was dose dependent; after 10 Gy the G_2/M delay was higher and longer for both cell lines (data not shown). The G_2/M delay in the radiosensitive IGRras was more pronounced and longer than for the IGRneo cell line with a peak at 16-22 hours (IGRras) and 15 hours (IGRneo) respectively (figure 1c). The IGRneo had fully recovered after 30 hours while the IGRras had not reached pre-treatment values by 36 hours.

Both for the neo and the ras transfected cell line the time for the RM to reach unity (the time that all cells have moved from the S phase compartment to the G_2/M phase) was the same (11 hours) (Figure 2a). Since the S phase percentage of either cell line was about the same, the cell cycle duration was not affected by transfection with the *NRAS* oncogene in untreated cells. After treatment with 5 Gy, the average slopes decreased compared with untreated cells for both cell lines (Figure 2b and 2c). However the difference in the slopes of the irradiated IGRneo and IGRras was not statistically different.

Discussion

In the present study a human melanoma cell line with increased radiosensitivity after transfection with mutated *NRAS* was tested to answer the question whether reduced survival correlated with altered cell cycle effects after irradiation. It was found that the radiosensitive IGRras cell line showed a longer G_2/M delay than the cell line containing the neomycin phosphotransferase gene only. Moreover, the duration of the S phase was not affected by transfection with *NRAS* and irradiation did not alter the duration of the S phase in either cell line.

Initial literature dealing with the relation between oncogene transfection and radiosensitivity using rat embryo fibroblasts [13,19,20,25,30], described an increased radioresistance after transfection with oncogenes *Hras* and *Myc*. Furthermore, these resistant cells showed a prolonged G_2/M delay [18] after radiation. It was postulated that in these cell lines the increased G_2/M delay would lead to improved repair of DNA damage, resulting in increased survival after radiotherapy [12,15].

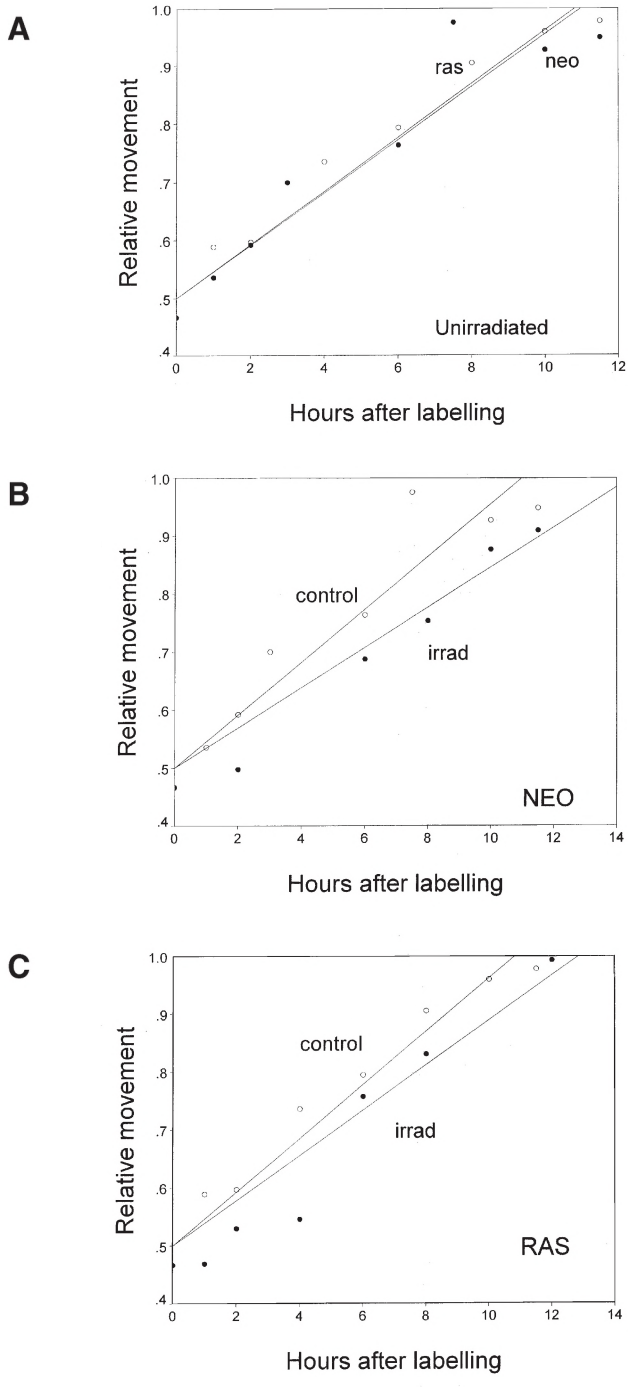


Figure 2

Relative movement at time intervals after labelling with BrdUrd for the two cell lines without irradiation (A) and after irradiation with 5 Gy versus their controls (IGRneo, B; and IGRras, C).

Based on our present observations and recent publications [22], we hypothesise that the prolongation of the G_2 phase is merely an expression of unrepaired damage. A strong argument in favour of this hypothesis is the observation [18] that after a higher irradiation dose the duration and the level of the G_2 phase even further increased. Moreover, ataxia telangiectasia (AT) cells [4,5,14], that are known for their high intrinsic radiosensitivity, show a prolonged G_2/M delay after irradiation. Ceraline [8], using normal human fibroblasts, also found that increased sensitivity was accompanied by a longer G_2/M delay. These observations are in agreement with data from Nagasawa et al. who observed that radiosensitive cell lines (human squamous cell carcinomas) responded to irradiation with a longer G_2 delay than radioresistant cell lines, when equal doses were given [22]. However using radiation doses which resulted in equal clonogenic survival for all cell lines, no difference in G_2 delay could be observed, showing the correlation between the survival and the duration of the G_2 delay.

Possible explanations for the discrepancies in literature dealing with the correlation between radioresistance and G_2 delay can be found in the origin and characteristics of the different cell lines used by the different research groups. The initial data in radiobiological literature mainly dealt with rodent embryo fibroblasts, a non-malignant cell type, expressing oncogenes obligatory for embryonic development. Recent publications on the relation between radioresistance and cell cycle effects using human cell lines indicate that G_2 delay did not always correlate with increased survival after radiation [3,16,29,31,36].

Warenius et al. [36] tested a panel of 12 human tumour cell lines with flow cytometry. In their study no clear relation between G_2 delay and radiosensitivity was found. However, the peak of cells in late G_1 accumulation, which is the G_1 peak arising when the number of cells held in G_2 /M starts to decrease, was predictive for radiosensitivity. In the present study, after irradiation, the number of cells in G_1 phase of the IGRras cell line recovers later than the IGRneo cell line (Fig 1a). We believe, however, that this late G_1 peak is only a reflection of the prolonged G_2 delay; after an initial decrease, the G_1 phase starts to rise more rapidly in the IGRneo cell line corresponding with a shorter G_2/M duration. Therefore, a late rise in G_1 would correspond with a higher intrinsic radiosensitivity.

Another important property of our cell line is that, in contrast with non-malignant rodent cell lines, this melanoma cell line contained mutated p53, which was probably, non-functional due to truncation. Chiarugi et al. [9] stated that the effect of dominant oncogenes on radiosensitivity is dependent on the p53 status of the cell line under investigation. Therefore, our observation that *NRAS* transfection caused increased radiosensitivity might due to the absence of wild type p53 in this cell line. In the present study the p53 status of the parental cell line explains the lack of accumulation of cells in G_1. Very recent literature has established also a role for p53 in the induction of G_2/M delay [3,7,10,23,28,37]. Bache [3] found that a reduction in G_2 delay correlated with increased apoptosis, although this phenomenon was not reflected in altered radiosensitivity. Guillouf [10] found that p53 was

responsible for a more rapid exit from G_2 delay, which was also correlated with increased levels of apoptosis. It can be suggested that cells with wild type p53 exhibit a reduced survival after radiation by increased apoptosis as a consequence of shortened G_2 delay. In cell lines expressing *Hras*, the incidence of radiation induced apoptosis is reduced compared to the parental cell line [17] while survival is simultaneously increased. Our melanoma cell line of human origin contains mutated p53. Therefore transfection with the oncogene *NRAS* may not have the same relative effect with regard to apoptosis inhibition. Consequently, the influence of N-ras in survival can not be predicted.

Since the S phase is important for DNA damage repair, we have tested whether after radiation differences exist in the duration of the S phase after radiotherapy, which might explain the observed differences in the duration of the G_2 delay. The duration of the S phase before treatment was exactly the same both for the ras transfected and the neo transfected cell line. After irradiation with a dose of 5 Gy, a small increase in the duration of the S phase was observed for both cell lines (Figure 2b and 2c). Also a small rise in number of cells in S phase is seen in the curves of Figure 1b. A possible interpretation of the shape of the curves (Figure 2b and 2c) is the existence of a lag period of around two hours after which the cell progress through S at the same rate as controls. This pattern is observed for both the neo and the ras cell line, and so if radiation induced differences in the duration of the S phase indeed occur, it will not provide an explanation for the difference in radiosensitivity.

It can be concluded that oncogenes may affect cell survival and the duration of the cell cycle. However, the influence of oncogenes on radiosensitivity is dependent on the origin and the characteristics of the cell line under investigation. Therefore, the prolongation of the G_2/M delay or oncogene expression is not sufficient to be used as a single parameter to predict intrinsic radiosensitivity.

Acknowledgements

The authors gratefully thank Dr. A. Begg, Dept. of Experimental Therapy, The Netherlands Kanker Institute, for his very fruitful and stimulating discussions.

Literature

1. Alapetite, C., Baroche, C., Remvikos, Y., Goubin, G. and Moustacchi, E. Studies on the influence of the presence of an activated *RAS* oncogene on the in vitro radiosensitivity of human mammary epithelial cells. Int. J. Radiat. Biol. 59: 385-396, 1991.

2. Aubert, C., Rouge, F. and Galindo, J.R. Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation in vitro. J. Nat. Cancer Inst. 64: 1029-1040, 1980.

3. Bache, M., Dunst, J., Wurl, P., et al. G_2/M checkpoint is p53-dependent and independent after irradiation in five human sarcoma cell lines. Anticancer Research 19: 1827-1832, 1999.

4. Beamish, H., Khanna, K.K. and Lavin, M.F. Ionizing radiation and cell cycle progression in ataxia telangiectasia. Radiat.Res. 138: S130-S133, 1994.

5. Beamish, H. and Lavin, M.F. Radiosensitivity in ataxia-telangiectasia - anomalies in radiation-induced cell cycle delay. Int. J. Radiat. Biol. 65: 1 75-184, 1994.

6. Begg, A.C., McNally, N.J., Shrieve, D.C. and Karcher, H. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. Cytometry 6: 620-626, 1985.

7. Bunz, F., Dutriaux, A., Lengauer, C., et al. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science 282: 1497-1501, 1998.

8. Ceraline, J., Deplanque, G., Duclos, B., et al. Relationships between p53 induction, cell cycle arrest and survival of normal human fibroblasts following dna damage. Bulletin du Cancer 84: 1007-1016, 1997.

9. Chiarugi, V., Magnelli, L., Cinelli, M., Turchetti, A. and Ruggiero, M. Dominant oncogenes, tumor suppressors, and radiosensitivity. Cell Mol. Biol. Res. 41: 161-166, 1995.

10. Guillouf, C., Rosselli, F., Krishnaraju, K., Moustacchi, E., Hoffman, B. and Liebermann, D.A. p53 involvement in control of G_2 exit of the cell cycle: role in DNA damage-induced apoptosis. Oncogene 10: 2263-2270, 1995.

11. Hermens, A.F. and Bentvelzen, P.A. Influence of the H-*ras* oncogene on radiation responses of a rat rhabdomyosarcoma cell line. Cancer Res. 52: 3073-3082, 1992.

12. Hwang, A. and Muschel, R.J. Radiation and the G_2 phase of the cell cycle. Radiat. Res. 150: S52-9, 1998.

13. Ling, C.C. and Endlich, B. Radioresistance induced by oncogenic transformation. Radiat. Res. 120: 267-279, 1989.

14. Lohrer, H.D. Regulation of the cell cycle following DNA damage in normal and Ataxia telangiectasia cells. Experientia 52: 316-328, 1996.

15. Maity, A., McKenna, W.G. and Muschel, R.J. The molecular basis for cell cycle delays following ionizing radiation: a review. Radiother. Oncol. 31: 1-13, 1994.

16. McIlwrath, A.J., Vasey, P.A., Ross, G.M. and Brown, R. Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res. 54: 3718-3722, 1994.

17. McKenna, W.G., Bernhard, E.J., Markiewicz, D.A., Rudoltz, M.S., Maity, A. and Muschel, R.J. Regulation of radiation-induced apoptosis in oncogene-transfected fibroblasts: influence of H-*ras* on the G_2 delay. Oncogene 12: 237-245, 1996.

18. McKenna, W.G., Iliakis, G., Weiss, M.C., Bernhard, E.J. and Muschel, R.J. Increased G_2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-*ras* and v-*myc*. Radiat.Res. 125: 283-287, 1991.

19. McKenna, W.G., Weiss, M.C., Bakanauskas, V.J., et al. The role of the *Hras* oncogene in radiation resistance and metastasis. Int. J. Radiat. Oncol. Biol. Phys. 18: 849-859, 1990.

20. McKenna, W.G., Weiss, M.C., Endlich, B., et al. Synergistic effect of the *myc* oncogene with *Hras* on radioresistance. Cancer Res. 50: 97-102, 1990.

21. Mendonca, M.S., Boukamp, P., Stanbridge, E.J. and Redpath, J.L. The radiosensitivity of human keratinocytes: influence of activated *c-H-ras* oncogene expression and tumorigenicity. Int. J. Radiat. Biol. 59: 1195-1206, 1991.

22. Nagasawa, H., Keng, P., Harley, R., Dahlberg, W. and Little, J.B. Relationship between gamma-ray-induced G_2 /M delay and cellular radiosensitivity. Int. J. Radiat. Biol. 66: 373-379, 1994.

23. Pellegata, N.S., Antoniono, R.J., Redpath, J.L. and Stanbridge, E.J. DNA damage and p53-mediated cell cycle arrest: a reevaluation. Proc. Natl. Acad. Sci.U.S.A. 93: 15209-15214, 1996.

24. Pomp, J., Ouwerkerk, I.J.M., Hermans, J., et al. The Influence of the Oncogenes *NRAS* and *MYC* on the Radiation Sensitivity of Cells of a Human Melanoma Cell line. Radiat. Res. 146: 374-381, 1996.

25. Samid, D., Miller, A.C., Rimoldi, D., Gafner, J. and Clark, E.P. Increased radiation

resistance in transformed and nontransformed cells with elevated ras proto-oncogene expression. Radiat. Res. 126: 244-250, 1991.

26. Schrier, P.I. Melanoma genetics and cytogenetics. Clin. Dermatol. 10: 31-39, 1992.

27. Schrier, P.I., Versteeg, R., Peltenburg, L.T.C., Plomp, A.C., Van 't Veer, L.J. and Krüse-Wolters, K.M. Sensitivity of melanoma cell lines to natural killer cells: a role for oncogene-modulated HLA expression? Semin. Cancer Biol. 2: 73-83, 1991.

28. Schwartz, D., Almog, N., Peled, A., Goldfinger, N. and Rotter, V. Role of wild type p53 in the G_2 phase - regulation of the gamma-irradiation-induced delay and DNA repair. Oncogene 15: 2597-2607, 1997.

29. Siles, E., Villalobos, M., Valenzuela, M.T., et al. Relationship between p53 status and radiosensitivity in human tumour cell lines. Br. J. Cancer 73: 581-588, 1996.

30. Sklar, M.D. The *ras* oncogenes increase the intrinsic resistance of NIH 3T3 cells to ionizing radiation. Science 239: 645-647, 1988.

31. Smeets, M.F., Mooren, E.H. and Begg, A.C. The effect of radiation on G_2 blocks, cyclin B expression and cdc2 expression in human squamous carcinoma cell lines with different radiosensitivities. Radiother. Oncol. 33: 217-227, 1994.

32. Su, L.N. and Little, J.B. Prolonged cell cycle delay in radioresistant human cell lines transfected with activated *RAS* oncogene and/or simian virus 40 T-antigen. Radiat. Res. 133: 73-79, 1993.

33. Van 't Veer, L.J., Burgering, B.M.T., Versteeg, R., et al. *NRAS* mutations in human cutaneous melanoma on sun-exposed body sites. Mol. Cell. Biol. 9: 3114-3116, 1989.

34. Van Elsas, A., Van Deursen, E., Wielders, R., van den Berg Bakker, C.A. and Schrier, P.I. *RAS* oncogene activation does not induce sensitivity to natural killer cell-mediated lysis in human melanoma. J. Invest. Dermatol. 103: 117S-121S, 1994.

35. Versteeg, R., Noordermeer, I.A., Krüse-Wolters, K.M., Ruiter, D.J. and Schrier, P.I. *MYC* downregulates class I HLA expression in human melanomas. EMBO J. 7: 1023-1029, 1988.

36. Warenius, H.M., Jones, M., Jones, M.D., Browning, P.G., Seabra, L.A. and Thompson, C.C.M. Late G_1 accumulation after 2 Gy of gamma-irradiation is related to endogenous raf-1 protein expression and intrinsic radiosensitivity in human cells. Br. J. Cancer 77: 1220-1228, 1998.

37. Winters, Z.E., Ongkeko, W.M., Harris, A.L. and Norbury, C.J. P53 regulates cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G_2 arrest in

human cells. Oncogene 17: 673-684, 1998.

CHAPTER IV

RADIOTHERAPY FOR OESOPHAGUS CARCINOMA: THE IMPACT OF P53 ON TREATMENT OUTCOME.

J. Pomp

J. Davelaar

J. Blom

C. van Krimpen

A.H. Zwinderman

W.G.V. Quint

J.J.F.M. Immerzeel

Reprinted from Radiotherapy and Oncology 46: 179-184, 1998
With permission from Elsevier Science

Abstract

Background and purpose: Wild type p53 protein plays an important role in the cellular response to ionizing radiation and other DNA damaging agents and is mutated in many human tumours. We evaluated the relationship of the immunohistochemically determined p53 protein status and the disease control with radiotherapy alone for carcinoma of the oesophagus. *Material and Methods*: Immunostaining for p53 protein was performed on paraffin-embedded specimens from 69 patients with adeno- and squamous cell carcinoma of the oesophagus. All patients were treated by radiotherapy exclusively, consisting of a combination of external irradiation combined with intraluminal brachytherapy, using two different dose levels. *Results*: 54% (37/69) of the tumours showed overexpression of the p53 protein. No difference in pre-treatment parameters for p53-positive and p53-negative cases was detected. In multivariate analysis p53 was significantly associated with Overall Survival (OS) next to weightloss, tumour stage and N-stage. For metastatic free survival (MFS) p53 status proved to be the sole independent prognostic factor. The influence of p53 on local recurrence free survival (LRFS) however, was not as strong as on OS and MFS. *Conclusions*; Immunohistochemically detected overexpression of mutated p53 protein in oesophagus carcinoma, was an independent prognostic factor in a group of patients, treated with radiotherapy alone.

Introduction

Treatment results of oesophageal carcinoma, with a mortality rate exceeding 80% at 2 years, have not been improved for the last decades. In case of locally advanced disease, without distant metastases, radiotherapy is given with palliative or sometimes curative intention. The introduction of chemotherapy in combination with either surgery or radiotherapy has some advantageous effects on survival [13,14], but further studies are needed in the future [18]. Death in oesophagus carcinoma is mostly related to local recurrence but also to distant failure.

P53 is a tumour suppressor gene, encoding for a nuclear phosphoprotein of 53 kilodalton (kDa). The protein product of this suppressor gene, wild type p53 protein, plays a role in the genomic stability, cell cycle control and DNA damage repair after treatment with ionizing radiation and cytotoxic agents in fibroblasts [21,22]. After DNA damage, wild type p53 is responsible for the cell cycle checkpoint at the G_1/S boundary, leading to cell cycle arrest in order to allow for DNA repair or to mediate apoptosis. Mutations in p53 lead to a prolonged half-life time and accumulation of the mutated protein in the nucleus. Furthermore the mutated p53 acts as an oncogene since accumulation of the mutated p53 product allows cells with damaged DNA to enter the cell cycle without arrest at end of the G_1 phase [16]. As DNA repair and apoptosis are prevented, mutated p53 is suggested to play a role in tumorigenesis [10]. The increased mutated p53 protein product can be detected by immunohistochemistry. Mutations of the p53 gene have been found in many tumours and amongst them the oesophagus [6,11,27]. It is described as an early event in oesophageal oncogenesis [24]. Clinical data suggest that the expression of p53 might predict worse response to treatment, and can serve as a prognostic factor in tumours [19,28]. Other studies, however, did not find a negative influence of p53 overexpression on treatment outcome [8,15,32].

We studied a group of patients with locally advanced oesophageal carcinoma, treated by radiotherapy exclusively, and determined the p53 protein expression by immunohistochemistry, in relationship to local control and survival.

Material and methods

Patients

At the Institute for Radiotherapy Friesland, The Netherlands, a prospective, non-randomised study was performed from 1987 to 1994, including 100 patients with inoperable cancer of the oesophagus. Patients with locally advanced tumours without distant metastases were treated with Radical Radiotherapy, with curative intention, consisting of External Beam RadioTherapy (EBRT) and Intra Luminal Brachytherapy (ILB) (patients in group I

were treated with 50 Gy EBRT plus 15 Gy ILB, i.e. the "low dose group", from 1987-1991 and patients in group II were treated with 60 Gy ERBT plus 12 Gy ILB, i.e. the "high dose group", from 1992-1994). Patients were prospectively scored for tumour stage, weight loss, dysphagia, tumour length, and histology. Tumour biopsies were obtained through endoscopy before treatment. Sixty-three patients had a CT scan and/or endoscopically performed ultrasound, and lymphnode status was obtained. No patients were known to have distant metastases at the time of diagnosis. From 69 tumours of these patients (Group I; n=41, group II; n=28) tissue blocks fixed in 4% formaldehyde and embedded in paraffin were available.

Immunohistochemistry

Tumour specimens were examined with regard to p53 protein expression. P53 protein was detected with the aid of immunoperoxidase, according to the Streptavidin Biotin Complex (SAB Complex) method, using the following antibody; p53 tumour suppressor Protein Ab-5 (Clone DO7), a BioGenex, mouse monoclonal antibody recognising mutant and wild type p53. D07 is a widely used antibody for immunohistochemistry (IHC) on formalin/paraffin tissues.

Sections of 4 μm thickness were cut from the paraffin embedded tissue blocks, and mounted on APES-coated (2% 3-Aminopropyltriethoxysilane, activated with 3% glutaraldehyde) slides. Slides were dewaxed in xylene and rehydrated in decreasing ethanol concentration to 70%, followed by endogenous peroxidase activity blocking for 20 minutes in 3% H_2O_2 in methanol and subsequently rinsed in demineralised water. Slides were pretreated for 10 minutes at 90°, in 0,1M Tris/EDTA at pH 8.0 (microwave oven/450 W). After cooling down for at least 20 minutes the slides were washed 2 minutes in demineralised water, 10 minutes in PBS, pre-incubated in 1% BSA/PBS for 10 minutes, followed by overnight incubation with the p53 antibody DO7 (dilution 1:400 in 1% BSA/PBS). Slides were washed 3 minutes in PBS, followed by 30 minutes incubating with Biotine labelled second antibody (Rabbit-anti-Mouse, DAKO, dilution 1:800 in 1% BSA/PBS). After washing for 3 minutes in PBS, slides were incubated for 30 minutes with SAB Complex-HRP (DAKO), washed 2 minutes in PBS before 10 minutes visualisation using diaminobenzidine (DAB), washed shortly in PBS and counterstained 3 minutes in Maier Haematoxylin. Finally slides were washed in running tap water, dehydrated and covered. As positive controls a breast- and colon-carcinoma were tested.

Analysis

The histopathologic diagnosis of each tumour was reviewed on separate HE stained slides. Determination of the p53 expression of tumour cells was done using a standardised procedure. The "tumour area" on the p53 stained biopsy slides was outlined by the surgical pathologist using a standard magnification of 100 x. The amount of p53 positive tumour

cells was scored semiquantitatively during light microscopy at a 400 x magnification (high powerfield). Since the tissue biopsies were small, the total area of the slides was examined (magnification 400 x) for p53 expression. The tumour was considered positive if at least 20% of the tumour cells expressed the protein. The level of 20% was chosen based on a paper by Cordon-Cordo et al [7], and also used by Wu et al [33], where it was established that a 20% cut-off correlates with mutations seen, when using other methods than IHC. We also assumed that a part of the cells would be in end G_1 with elevated wild type protein. The p53 status was scored by 2 of the authors independently. Discrepancies were discussed until agreement was reached.

The correlation was studied between p53 expression and clinical parameters such as histology, length of the tumour and stage. Overall survival (OS) and freedom from local recurrence as well as freedom from distant metastasis was calculated for both p53- and p53+ tumours, using the Kaplan Meier method. The log-rank test was used to test the difference between the curves.

In multivariate Cox regression analysis, the effects of confounders (age, sex, weightloss, topography, length of tumour, histology, stage and N-classification and treatment dose) on survival was modelled in a forward stepwise manner. Thereafter, p53 status was forced into the model and tested for significance.

Results

Histology and immunohistochemistry

Twenty-five tumours were squamous cell carcinomas, 43 were adenocarcinomas and 1 tumour was classified as anaplastic. Fifty four percent of the tumours (37/69) had overexpression of p53 protein after staining with DO7. Of the squamous cell carcinomas 17/25 (68%) stained positively for p53 while in the adenocarcinoma group this was 20/43 (47%). The anaplastic tumour showed no p53 overexpression. P53 expression was not related to age, sex, histology, tumour site, lymphnode status and tumour length (Table 1).

Correlation between treatment results and p53 expression

The follow up range was 2-5 years in the high dose group and 5-9 years in the low dose group. For statistical analysis, only the group of tumours were used of which p53 status was determined. Six patients are still alive without tumour, 63 are dead of whom 19 with local recurrence only, 15 with metastasis only, and 11 failed both locally and at distant. Eighteen persons have died without the presence of tumour.

When comparing the p53 negative (p53-) and p53 positive (p53+) tumours, a significant difference in OS ($p=0.033$) was seen (figure 1), with a median survival of 11,5 and 7,3 months respectively. When stratified for total dose, the difference between p53- and p53+

Table 1
Relationship between p53 staining and pretreatment variables.

	p53 +	p53-	p-value
Age			
≤ 65 years	25	21	
> 65 years	12	11	n.s
Sex			
Male	28	27	
Female	9	5	n.s
Weightloss			
No weight loss	6	10	
0-5 kilo	9	8	
6-10 kilo	9	4	
> 10 kilo	8	7	n.s
Unknown	5	3	
Topography			
superior part	1	2	
middle part	8	6	
lower part	28	24	n.s.
Length			
0-30 mm	4		
30-70 mm	22	23	
> 70 mm	11	9	n.s
Histology			
squamous cell carcinoma	17	8	
adenocarcinoma	20	23	
anaplastic	1	n.s	
Stage			
I	4		
II	21	23	
III	5	3	
IV	7	6	n.s
N-stage			
N0	24	17	
N+	11	11	
Nx	2	4	n.s
Treatment Dose			
50 + 15 Gy	22	19	
60 + 12 Gy	15	13	n.s

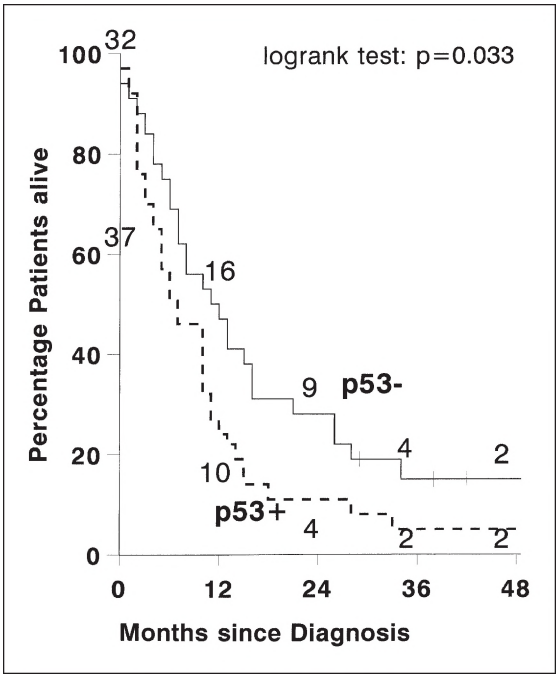
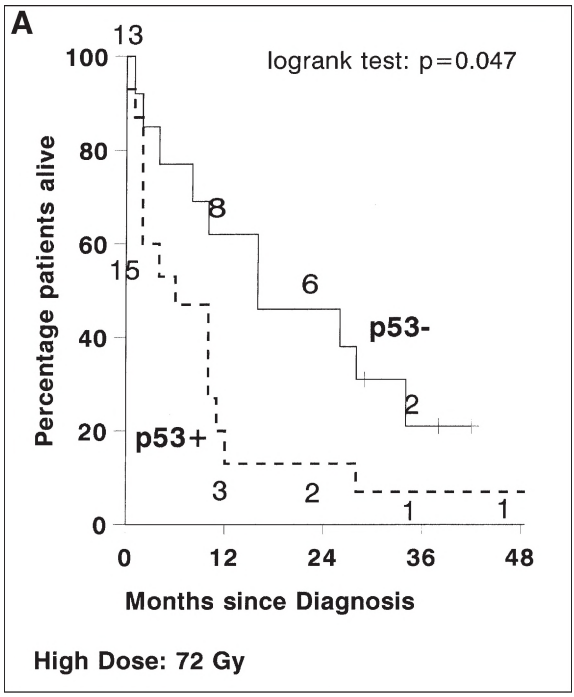


Figure 1
Overall survival for p53- (———) and p53+ (- - - - -) tumours, when the whole group is considered.

tumours in survival remains in the 72 Gy group (p=0.047) (figure 2a), while in the low dose group (65 Gy) no difference in OS was found between p53+ and p53- tumours (p=0.39) (figure 2b). For all patients, in multivariate analysis, confounding factors of mortality were weight loss, tumour stage and N-stage. P53 status was still significantly associated with mortality (p=0.014). The unadjusted hazard rate was 1.72 (95% confidence interval 1.04-2.85), and after adjustment the hazard rate was 1.96 (c.i. 1.15 - 3.31). With regard to local recurrence free survival, we found no influence of p53 status for the whole group (p=0.19). When stratified for total dose we found in the group receiving 72 Gy an indication that the local recurrence rate was reduced in the p53- cases compared to the p53+ cases, with a median local recurrence free survival of 20 and 12 months respectively. Using the log-rank statistic this was not significant (p=0.13), but when using the Breslow statistic, which weighs early events more importantly than late events, this was significant (p=0.033). Local recurrence free survival was not associated with the confounders in Table 1 ($p > 0.06$). A strong correlation between the development of distant metastases and p53 expression was found. The p53- group had a higher distant metastasis-free survival with a p-value of 0.0097 (Figure 3). This was dose-independent. In multivariate analysis, none of the confounders mentioned in Table 1 was found to be related to metastasis free survival ($p > 0.22$), and consequently p53 status was the sole independent prognostic factor.



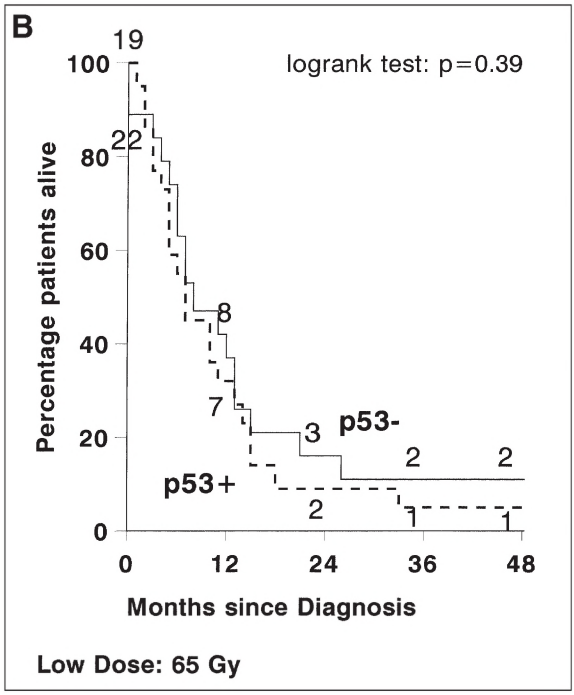


Figure 2
Overall survival for p53- (———) and p53+ (- - - - -) tumours, when stratified for high dose group (Figure 2a) and low dose (Figure 2b).

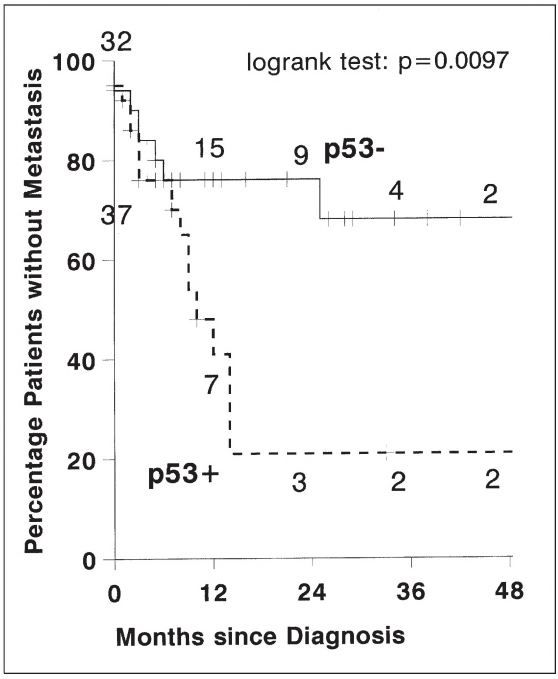


Figure 3
Distant metastases free survival for p53- (———) and p53+ (- - - - -) tumours.

Discussion

P53 is a proto-oncogene, responsible for cell cycle control, growth differentiation and response after cytotoxic treatment. It is involved in the G_1 block after radiotherapy and regulates apoptotic response [5,16]. When mutated, p53 acts as an oncogene. As a consequence the G_1 cell cycle response after cytotoxic treatment is absent and apoptosis does not occur [21]. This is held responsible for the increased radioresistance, observed mostly *in vitro*, [16,20,23] although not always confirmed by others [2,29,34].

For oesophageal carcinoma, surgery is the treatment of first choice. Survival however, even after complete resection is very disappointing [30]. For inoperable oesophagus carcinoma, radiotherapy with curative intention is considered for selected patients.

As the p53 gene plays a role in the response to ionizing radiation we investigated in a group of patients with locally advanced carcinoma of the oesophagus, treated with radiotherapy alone, whether mutation of p53 influences treatment outcome. *In vitro* data strongly suggest that p53 mutations lead to increased radioresistance, therefore it is of interest to know whether p53 status of tumours can be of predictive value for radiocurability. We looked at overall survival, local control and the occurrence of distant metastases. It was found that p53 is an independent predictive factor for the overall survival and the occurrence

of distant metastases. The influence on locoregional control was less evident for the whole duration of follow up, however for locoregional failure as an early event it was a significant factor.

Several authors have reported about mutated p53 as a prognostic factor in oesophagus carcinoma, however this mostly concerned patients who underwent surgery as part of the treatment. While some authors found no influence of p53 on survival [25,26,31], others found impaired [4] or better [27] prognosis with mutated p53.

Clinical studies correlating outcome after radiotherapy with p53 status for a large series of tumours are reviewed in a paper by Bristow et al. [3]. It was concluded that up till now, it remains unclear whether p53 can serve as a predicting factor for outcome after radiotherapy.

Several arguments for the lack of correlation between p53 status and treatment results for oesophageal carcinoma can be put forward. Firstly, immunohistochemically determination of the p53 status has a number of limitations; wild type p53, for example, can also show an increased expression which is not a feature of mutated protein in this case [12]. In contrast, p53 can be mutated without being detected by IHC as in the case of nonsense, splicing variants and stop-codons mutations [1]. As we did not perform sequence analysis, indeed per sample we can not exclude that normal expression did not hide a mutation, not picked up by IHC or visa versa that p53 positive staining was the overexpression of abnormally abundant but normally functioning protein [17]. The fixation of the archive material and the choice of antibodies play a crucial role too [9]. We chose D07 as this is a widely used antibody, so that our study is comparable to others in literature. Secondly p53 mutation alone might not be enough to influence treatment outcome, as carcinogenesis is a multistep process and many more oncogenes are involved, each of them with a possible influence on treatment outcome. Thirdly, the number of patients may not be sufficiently large enough to determine any difference between the 2 groups. We investigated the influence of p53 as a predictive factor for treatment outcome after radiotherapy of oesophagus carcinoma. In our series, consisting of 69 patients with locally advanced tumours, immunohistochemically detection of p53 was not correlated to pre-treatment parameters. We conclude that in this group of patients, p53 status as determined by IHC, did not seem to predict radiosensitivity, but that it served as an independent prognostic factor for overall survival and the occurrence of distant metastases.

Acknowledgements:

The generous help of Dr. H.F. Eggink, pathologist in Leeuwarden for the provision of all the tissue blocks is gratefully acknowledged. We would like to thank Dr. P. Spoelstra, gastro-enterologist, Leeuwarden for the carefully performed endoscopies before treatment

and during follow up and the co-operation during the Intraluminal Brachytherapy. The data management carried out by Ms. K.J. v.d. Elsaker is highly appreciated.

Literature

1. Bodner, S.M., Minna, J.D., Jensen, S.M., et al. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. Oncogene 7: 743-749, 1992.

2. Brachman, D.G., Beckett, M., Graves, D., Haraf, D., Vokes, E. and Weichselbaum, R.R. p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines [see comments]. Cancer Res. 53: 3667-3669, 1993.

3. Bristow, R.G., Benchimol, S. and Hill, R.P. The p53 gene as a modifier of intrinsic radiosensitivity -Implications for radiotherapy [Review]. Radiother. Oncol. 40: 197-223, 1996.

4. Chanvitan, A., Nekarda, H. and Casson, A.G. Prognostic value of DNA index, S-phase fraction and p53 protein accumulation after surgical resection of esophageal squamous-cell carcinomas in Thailand. Int. J. Cancer 63: 381-386, 1995.

5. Clarke, A.R., Purdie, C.A., Harrison, D.J., et al. Thymocyte apoptosis induced by p53-dependent and independent pathways [see comments]. Nature 362: 849-852, 1993.

6. Coia, L.R. and Sauter, E.R. Esophageal cancer. Curr. Probl. Cancer 18: 189-247, 1994.

7. Cordon Cardo, C., Dalbagni, G., Saez, G.T., et al. p53 mutations in human bladder cancer: genotypic versus phenotypic patterns. Int. J. Cancer 56: 347-353, 1994.

8. Esrig, D., Elmajian, D., Groshen, S., et al. Accumulation of nuclear p53 and tumor progression in bladder cancer [see comments]. N. Engl. J. Med. 331: 1259-1264, 1994.

9. Fisher, C.J., Gillett, C.E., Vojtesek, B., Barnes, D.M. and Millis, R.R. Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. Br. J. Cancer 69: 26-31, 1994.

10. Fung, C.Y. and Fisher, D.E. p53: from molecular mechanisms to prognosis in cancer [editorial]. J. Clin. Oncol. 13: 808-811, 1995.

11. Galiana, C., Lozano, J.C., Bancel, B., Nakazawa, H. and Yamasaki, H. High frequency of Ki-ras amplification and p53 gene mutations in adenocarcinomas of the human esophagus. Molecular Carcinogenesis 14: 286-293, 1995.

12. Hall, P.A. and Lane, D.P. p53 in tumour pathology: can we trust immunohistochemistry? Revisited! [editorial] [see comments]. J. Pathol. 172: 1-4, 1994.

13. Haller, D.G. Treatments for esophageal cancer [editorial; comment]. N. Engl. J. Med. 326: 1629-1631, 1992.

14. Herskovic, A., Martz, K., al Sarraf, M., et al. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus [see comments]. N. Engl .J. Med. 326: 1593-1598, 1992.

15. Jansson, T., Inganas, M., Sjogren, S., et al. p53 status predicts survival in breast cancer patients treated with or without postoperative radiotherapy: A novel hypothesis based on clinical findings. J. Clin. Oncol. 13: 2745-2751, 1995.

16. Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311, 1991.

17. Kocialkowski, S., Pezzella, F., Morrison, H., et al. Mutations in the p53 gene are not limited to classic 'hot spots' and are not predictive of p53 protein expression in high-grade non-Hodgkin's lymphoma. Br. J. Haematol. 89: 55-60, 1995.

18. Kok, T.C. and Tilanus, H.W. Neoadjuvant treatment in oesophageal cancer: the needs for future trials. The Rotterdam Esophageal Tumor Study Group. Eur. J. Surg. Oncol. 22: 323-325, 1996.

19. Langendijk, J.A., Thunnissen, F.B.J.M., Lamers, R.J.S., De Jong, J.M.A., Ten Velde, G.P.M. and Wouters, E.F.M. The prognostic significance of accumulation of p53 protein in stage III non-small cell lung cancer treated by radiotherapy. Radiother. Oncol. 36: 218-224, 1995.

20. Lee, J.M. and Bernstein, A. p53 mutations increase resistance to ionizing radiation. Proc. Natl. Acad. Sci.USA 90: 5742-5746, 1993.

21. Lowe, S.W., Ruley, H.E., Jacks, T. and Housman, D.E. P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957-967, 1993.

22. Maity, A., McKenna, W.G. and Muschel, R.J. The molecular basis for cell cycle delays following ionizing radiation: a review. Radiother. Oncol. 31: 1-13, 1994.

23. McIlwrath, A.J., Vasey, P.A., Ross, G.M. and Brown, R. Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res. 54: 3718-3722, 1994.

24. Parenti, A.R., Rugge, M., Frizzera, E., et al. p53 overexpression in the multistep process of esophageal carcinogenesis. Am. J. Surg. Pathol. 19: 1418-1422, 1995.

25. Puglisi, F., Diloreto, C., Panizzo, R., et al. Expression of p53 and bcl-2 and response to preoperative chemotherapy and radiotherapy for locally advanced squamous cell carcinoma of the oesophagus. J. Clin. Pathol. 49: 456-459, 1996.

26. Sarbia, M., Porschen, R., Borchard, F., Horstmann, O., Willers, R. and Gabbert, H.E. p53 protein expression and prognosis in squamous cell carcinoma of the esophagus. Cancer 74: 2218-2223, 1994.

27. Sauter, E.R., Keller, S.M. and Erner, S.M. P53 correlates with improved survival in patients with esophageal adenocarcinoma. J. Surg. Oncol. 58: 269-273, 1995.

28. Shin, D.M., Lee, J.S., Lippman, S.M., et al. p53 expression: Predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. J. Natl. Cancer Inst. 88: 519-529, 1996.

29. Slichenmyer, W.J., Nelson, W.G., Slebos, R.J. and Kastan, M.B. Loss of a p53-associated G_1 checkpoint does not decrease cell survival following DNA damage. Cancer Res. 53: 4164-4168, 1993.

30. Tilanus, H.W. Changing patterns in the treatment of carcinoma of the esophagus. Scand. J. Gastroenterol. 30: 38-42, 1995.

31. Vijeyasingam, R., Darnton, S.J., Jenner, K., Allen, C.A., Billingham, C. and Matthews, H.R. Expression of p53 protein in oesophageal carcinoma: clinico-pathological correlation and prognostic significance. Br. J. Surg. 81: 1623-1626, 1994.

32. Wilson, G.D., Richman, P.I., Dische, S., et al. p53 status of head and neck cancer: relation to biological characteristics and outcome of radiotherapy. Br. J. Cancer 71: 1248-1252, 1995.

33. Wu, C.S., Pollack, A., Czerniak, B., et al. Prognostic value of p53 in muscle-invasive bladder cancer treated with preoperative radiotherapy. Urology. 47: 305-310, 1996.

34. Zaffaroni, N., Benini, E., Gornati, D., Bearzatto, A. and Silvestrini, R. Lack of a correlation between p53 protein expression and radiation response in human tumor primary cultures. Stem.Cells Dayt. 13: 77-85, 1995.

CHAPTER V

E-CADHERIN EXPRESSION IN OESOPHAGEAL CARCINOMA TREATED WITH HIGH DOSE RADIOTHERAPY; CORRELATION WITH PRE-TREATMENT PARAMETERS AND TREATMENT OUTCOME.

J. Pomp

J. Blom

C. van Krimpen

A.H. Zwinderman

J.J.F.M. Immerzeel

Reprinted from: J. Cancer Res. Clin. Oncol. 125: 641-645, 1999
With permission from Springer-Verlag

Abstract

Background and purpose: E-cadherin plays an important role in the cell-cell contact of normal epithelium. Loss of E-cadherin expression may be related to tumour invasiveness and metastatic potential. In a group of patients treated for oesophageal carcinoma by radiotherapy only, we found that immunohistochemically detected p53 expression correlated with reduced survival, mainly due to the occurrence of distant metastases. We questioned whether in this group of patients, E-cadherin expression was concomitantly altered and served as a predictive factor for the development of distant metastases. *Materials and Methods*: Immunostaining for E-cadherin was performed on paraffin-embedded biopsy specimens from patients with adenocarcinoma and squamous cell carcinoma of the oesophagus. E-cadherin status and its correlation with regard to pre-treatment parameters and treatment outcome were determined. *Results*: An aberrant staining pattern of E-cadherin did not correlate with any of the pre-treatment parameters. In a univariate analysis, a significantly reduced metastatic potential was found for tumours that had an aberrant cellular staining pattern for E-cadherin, which was strongest for squamous cell carcinomas. However, in a multivariate analysis only p53 status correlated significantly with the occurrence of distant metastases. *Conclusion*: Although in univariate analysis, aberrant E-cadherin expression served as a better, rather than a worse prognostic factor, p53 status remained the only significant parameter in multivariate analysis, in this group of patients with oesophageal carcinoma. No relationship between p53 status and E-cadherin expression was found.

Introduction

E-cadherin is a transmembrane glycoprotein that mediates cell adhesion and plays a role in the cell-cell contact of normal epithelium [1]. Its presence can be detected by immunohistochemical techniques. Its gene maps to chromosome 16q22, and the product of the gene is involved in embryogenesis and organogenesis by mediating epithelial cell-cell recognition and adhesion [8,12]. Cadherins are bound to intracellular proteins, the catenins [1,3,4]. E-cadherin expression is altered or reduced in many tumours leading to detachment of cancer cells, facilitating the formation of metastases [4,10].

In one study [6], it was found that premalignant skin lesions in p53-knock out mice showed alterations in E-cadherin expression, while this was rarely the case in tumours with heterozygote or wild type p53. Another clinical study [5] showed an association between down regulation of E-cadherin expression and alteration in p53 in a series of 210 breast carcinomas, suggesting that p53 could play a role in the regulation of E-cadherin expression. A further study [7] also found a correlation between reduced E-cadherin expression and positive p53 staining in breast carcinomas. Two recent papers have shown that radiation may increase E-cadherin expression in human tumour cell systems [2,9], suggesting that radiation may influence the metastatic behaviour of tumours. In a recent study in oesophageal carcinoma treated exclusively with radiotherapy, we found that p53 overexpression was correlated with reduced overall survival and the increased occurrence of distant metastases [16].

We therefore questioned whether E-cadherin could be a prognostic factor for tumours treated by radiotherapy, and whether this is related to p53 status, indicating a possible functional link between E-cadherin and p53 status in radiation response. As a model we took oesophageal tumours, irradiated with high dose. In this group of patients, p53 status had been shown to correlate with overall survival (OS) by increased distant metastases.

Material and methods

Patients

In a group of 65 patients with locally advanced carcinoma of the oesophagus treated between 1987 and 1994 by radiotherapy only, paraffin embedded archival material from pre-treatment biopsies, obtained by endoscopic procedures was available to test the E-cadherin expression by immunohistochemistry.

The radiotherapy consisted of external radiation to 50 Gy (from 1987-1992) or 60 Gy (from 1992-1994), with an intraluminal boost of two times 7.5 Gy or 2 times 6 Gy respectively. The opinion, based on literature, that 50 Gy was insufficient to treat the regional lymph nodes adequately lead to an increase of the external dose delivered, with a concomi-

tant reduction of the brachytherapy dose to avoid acute and late toxicity. All patients had locally extensive disease without distant metastases and were suitable to undergo high dose radiotherapy. All patients were prospectively followed and questionnaires were filled-out every visit.

Immunohistochemistry.

E-cadherin expression of the tumour specimens was detected by immunohistochemistry and visualised using the Streptavidin Biotin Complex (SAB Complex) method. The monoclonal mouse anti-E-cadherin antibody HECD-1 (Takara, Berkeley, CA) was used at a dilution of 1:400.

Section of 4 μm were cut from the paraffin embedded tissue blocks, and mounted on APES-coated slides (2% 3-Aminopropyltriethoxysilane, activated with 3% glutaraldehyde). Slides were dewaxed in xylene and rehydrated in decreasing ethanol concentration to 70%, followed by endogenous peroxidase activity blocking for 20 minutes in 3% H_2O_2 in methanol and subsequently rinsed in demineralised water. Slides were pre-treated for 10 minutes at 90°, in 0,1M Tris/EDTA at pH 8.0 (microwave oven/450 W). After cooling for at least 20 minutes the slides were washed for 2 minutes in demineralised water, 10 minutes in PBS, pre-incubated in 1% BSA/PBS for 10 minutes, followed by overnight incubation with the antibody HECD-1 (dilution 1:400 in 1% BSA/PBS). Slides were washed 3 minutes in PBS, followed by 30 minutes incubation with biotine-labelled second antibody (rabbit-anti-mouse, DAKO, dilution 1:300 in 1% BSA/PBS). After washing for 3 minutes in PBS, slides were incubated for 30 minutes with horseradish-peroxidase-linked streptavidin-biotin complex (DAKO), and washed for 2 minutes in PBS. To detect the labelling, incubation for 10 minutes with diaminobenzidine was used. After the slides had been washed briefly in PBS, they were counterstained for 3 minutes with Mayer Haematoxylin. Finally the slides were washed in running tap water, dehydrated and covered.

Analysis

The whole biopsy area as presented on the slide was scored for E-cadherin expression in the areas containing the malignant cells. E-cadherin staining was scored as normal or abnormal (N/A). Cytoplasmic, apical, aberrant, absent and basal staining of tumour cells were considered abnormal. If at least 10% of the cells expressed E-cadherin in this way, the tumour was considered to have an abnormal staining pattern [17,19]. Staining of the lateral plasma membrane was considered normal. Non-malignant epithelium assessed on the same slide served as an internal control.

E-cadherin expression was correlated with clinical parameters such as age, sex, weight loss, topography, length of tumour, histology, p53 status, stage and N-classification. Overall survival and freedom from local recurrence as well as freedom from distant metastases were assessed for tumours with normal versus abnormal expression of E-cadherin, using the

Kaplan-Meier method. The log-rank test was used to test for the differences.

In a multivariate Cox regression analysis, the effects of confounders (age, sex, weight loss, topography, length of tumour, histology, grade, stage, p53 status, N-classification and treatment dose) on survival was modelled in a forward stepwise manner.

Results

Patients

At the time of evaluation with a follow up period of 1-6 years, 6 patients were still alive (more than 3 years), and 59 had died of whom 18 with local recurrence only, 13 with distant metastases only and 10 with both local and distant failure. Eighteen patients had died without tumour.

Analysis

Fifty-one tumours (78%) expressed E-cadherin abnormally while 22% showed normal expression of E-cadherin on the lateral plasma membrane. In Table 1 the correlation of the pre-treatment parameters with the expression of E-cadherin is shown. E-cadherin expression did not correlate with any of the 11 pre-treatment parameters tested.

E-cadherin expression did not predict the outcome of treatment with regard to local recurrence-free survival or overall survival (Table 2). However, distant metastases occurred less frequently when E-cadherin was expressed abnormally (Figure 1, p=0.033): The relative risk was 2.47 (95 % c.i. 1.05-5.8). After correcting for the pre-treatment p53 status as a confounder, the relative risk was reduced to 1.45 (95% c.i. 0.52-4.09). The only significant prognostic factor in multivariate analysis was the p53 status (p=0.037). Stratification according to tumour histology (adenocarcinoma versus squamous cell carcinoma) showed that abnormal E-cadherin expression correlated significantly with a longer metastatatic free period (MFP) for squamous cell carcinomas (p=0.0256). In the group of adenocarcinomas the influence of E-cadherin expression on MFP was not significant (p=0.29) (Figure 2a and 2b). Overall survival in both histology groups was not influenced by the E-cadherin status.

The total dose delivered neither influenced the disease free survival (p=0.99) nor the distant metastases free survival (p=0.98). The effect of E-cadherin expression on survival without distant metastases was also the same in both dose groups.

Discussion

In a previous study it was found that p53 positive oesophageal tumours treated by radiotherapy meant a significantly reduced overall survival, which was due to the increased

Tabel 1
Correlation between e-cadherin expression and pre-treatment parameters

	Abnormal expression (n=51)		Normal expression (n=14)	
Adenocarcinoma	30	(59%)	10	(71%)
P53 positive	23	(46%)	8	(73%)
Grade III	30	(60 %)	4	(29%)
Stage I,II	35	(69%)	11	(78%)
Total Dose 72	5	(10%)	2	(14 %)
Age (mean)	69 yrs	(+/- 9)	67 yrs	(+/- 10)
Nodal stage N+	19	(37%)	4	(29%)
Nodal stage N0	27	(54 %)	9	(64 %)
Nodal stage Nx	5	(10%)	1	(7%)
Male	41	(80%)	13	(93%)
Upper third	3	(6%)	0	(0%)
Middle third	11	(22%)	4	(28%)
Lower third	37	(72%)	10	(71 %)
Length (mean)	64 mm	(+/- 25)	64 mm	(+/- 22)
Weightloss (mean)	4.9 Kg	(+/- 4.7)	7.0	(+/- 8.0)

No difference was statistically significant.

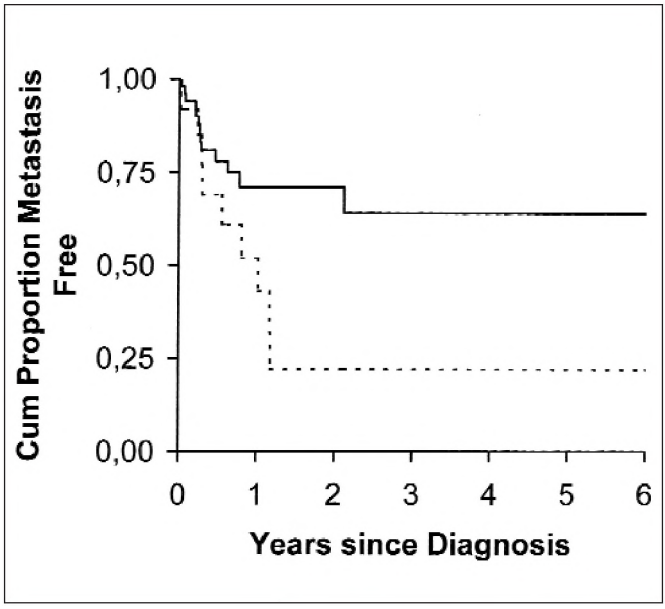


Figure 1
Distant metastases free survival when E-cadherin stains normal (- - - - -) or abnormal (———) ($p<0.05$).

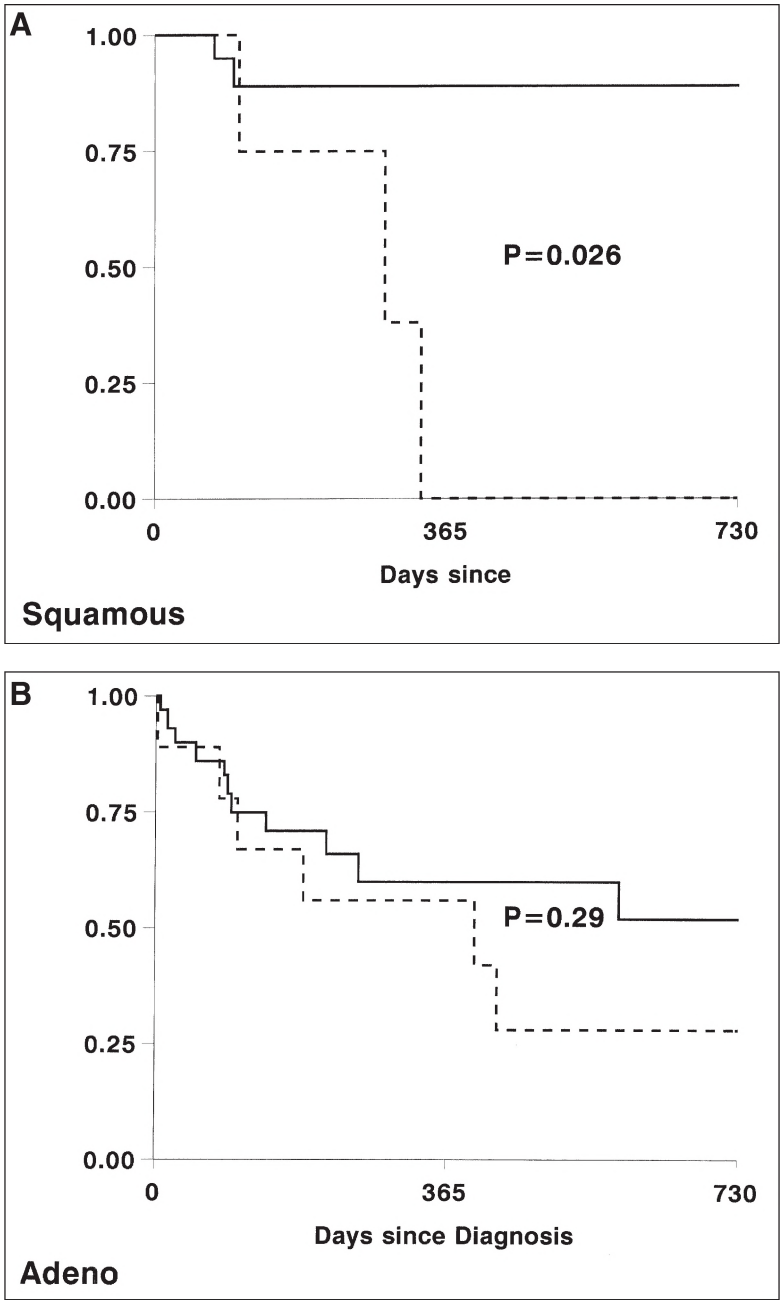


Figure 2
Distant metastases free survival when E-cadherin stains normal (- - - - -) or abnormal (———) for squamous cell carcinomas (Fig. 2a) or Adenocarcinomas (Fig 2b)

Table 2
E-cadherin expression and treatment outcome

		a (n=51)	n (n=14)	P*
Survival: % (SE)	6 mths	61 (7)	79 (11)	0.91
	1 year	31 (7)	50 (13)	
RFP: % (SE)	6 mths	67 (7)	73 (13)	0.91
	1 year	57 (8)	55 (15)	
RFS: % (SE)	6 mths	45 (7)	62 (13)	0.68
	1 year	24 (6)	23 (12)	
MFP: % (SE)	6 mths	78 (6)	69 (13)	0.033
	1 year	71 (8)	52 (14)	
MFS: % (SE)	6 mths	55 (7)	62 (13)	0.96
	1 year	27 (6)	46 (14)	
DFS: % (SE)	6 mths	41 (7)	46 (14)	0.98
	1 year	22 (6)	23 (12)	

* p value of Student's t-test, Pearson chi-square, or logrank test, where appropriate.

a: abnormal expression
n: normal expression

RFP (relapse free period), RFS (relapse free survival), MFP (metastasis free period) MFS (metastasis free survival) DFS (disease free survival)

occurrence of distant metastases [16]. Since E-cadherin is a marker for tumour invasiveness and the occurrence of distant metastases, we investigated whether E-cadherin expression was related to pre-treatment parameters, including p53 status and whether it could also serve as a prognostic factor. No correlation of E-cadherin expression with any of the pre-treatment parameters tested, including p53 status, was found. A reduced probability of distant metastases was found in cases showing an aberrant staining pattern of E-cadherin. Stratifying according to histology showed that this was significant for squamous cell carcinomas. In a multivariate analysis, only p53 correlated significantly with the development of distant metastases and overall survival.

Reduced expression of E-cadherin in oesophageal carcinoma has been described before, and correlated with high stage, high histological grade and reduced survival [14,15,18]. The worse prognosis with reduced E-cadherin expression was often explained by the elevated

risk of distant metastases when cell-cell contact is disturbed. Only one study [11] found an opposite relationship between abnormal E-cadherin expression and the occurrence of liver metastases in pancreatic tumours, i.e. the cells that expressed E-cadherin normally had increased chance to form metastases.

We found that abnormal E-cadherin expression correlated with distant metastases only for squamous cell carcinomas. A similar result has been published previously [13] although in that paper absent E-cadherin expression served as a worse prognostic factor. To explain the inconsistent correlation between immunohistochemically detected E-cadherin expression in tumours and the development of distant metastases, several arguments can be put forward. First, the terminology used for the immunohistochemical expression of E-cadherin is not consistent throughout the literature; either the level of expression is looked at (normal versus reduced) or both the level and the localisation (abnormal versus normal expression). Although in theory detachment of cancer cells would be facilitated by abnormal E-cadherin function, normal expression may be necessary in order to establish metastases [11].

E-cadherin expression can be increased by radiation, as described in recent papers [2,9]. If so, and if E-cadherin is also involved in the establishment of distant metastases, it may be hypothesised that radiotherapy may influence metastatic potential of tumour cells during treatment. This could be another possible explanation why E-cadherin can be of different prognostic importance in tumours treated by radiotherapy only, compared to tumours that are treated surgically.

The concept that ionising radiation may modify metastatic potential of tumours is not new. In a review by von Essen [20], the influence of local radiotherapy on the occurrence of distant metastases, both in vivo experiments and clinical data are extensively described. These are all mostly descriptive papers, however, and no study correlating radiotherapy and distant metastases looked for gene or protein expression that can mediate this phenomenon . In a multivariate analysis, only p53 status correlated with distant metastases. Any E-cadherin effect, therefore, could be masked by the p53 status. Although we found no correlation between p53 status and E-cadherin status, this study can not rule out a direct influence of p53 on E-cadherin expression.

So far one study in rodents [6] has shown a, presumably functional, correlation between the p53 status and E-cadherin expression and two clinical studies have found a correlation between p53 status and E-cadherin expression [5,7].

In summary, we found in a group of 65 patients treated by high dose radiotherapy for locally advanced oesophageal carcinoma that aberrant expression of E-cadherin correlated with reduced occurrence of distant metastases, particularly for squamous cell carcinomas. In multivariate analysis, however, only p53 status correlated with the occurrence of distant metastases. We could not establish a relationship between p53 status and E-cadherin expression in this series.

Acknowledgements

The generous help of Dr. H.F. Eggink, pathologist in Leeuwarden for the provision of all the tissue blocks is gratefully acknowledged. We would like to thank Dr. P. Spoelstra, gastro-enterologist, Leeuwarden for the carefully performed endoscopies before treatment and during follow up and the co-operation during the Intraluminal Brachytherapy. The data management carried out by Ms. K.J. v.d. Elsaker is highly appreciated. We are grateful to Dr. A. Begg, The National Cancer Institute, Amsterdam, for the reviewing of the manuscript and the fruitful suggestions.

Literature

1. Aberle, H., Schwartz, H. and Kemler, R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. J.Cell Biochem. 61: 514-523, 1996.

2. Akimoto, T., Mitsuhashi, N., Saito, Y., Ebara, T. and Niibe, H. Effect of radiation on the expression of e-cadherin and alpha-catenin and invasive capacity in human lung cancer cell line in vitro. Int. J. Radiat. Oncol., Biol., Phys. 41: 1171-1176, 1998.

3. Balsamo, J., Leung, T., Ernst, H., Zanin, M.K., Hoffman, S. and Lilien, J. Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. J. Cell Biol. 134: 801-813, 1996.

4. Bracke, M.E., Van Roy, F.M. and Mareel, M.M. The E-cadherin/catenin complex in invasion and metastasis. Curr. Top. Microbiol. Immunol. 213: 123-161, 1996.

5. Bukholm, I.K., Nesland, J.M., Karesen, R., Jacobsen, U. and Borresen Dale, A.L. Expression of E-cadherin and its relation to the p53 protein status in human breast carcinomas. Virchows Arch. 431: 317-321, 1997.

6. Cano, A., Gamallo, C., Kemp, C.J., et al. Expression pattern of the cell adhesion molecules. E-cadherin, P-cadherin and alpha 6 beta 4 intergrin is altered in pre-malignant skin tumors of p53-deficient mice. Int. J. Cancer 65: 254-262, 1996.

7. Charpin, C., Garcia, S., Bouvier, C., et al. E-cadherin quantitative immunocytochemical assays in breast carcinomas. J. Pathol. 181: 294-300, 1997.

8. Dobson, H., Pignatelli, M., Hopwood, D. and D'Arrigo, C. Cell adhesion molecules in oesophageal epithelium. Gut 35: 1343-1347, 1994.

9. Ebara, T., Mitsuhashi, N., Saito, Y., Akimoto, T. and Niibe, H. Change in e-cadherin expression after x-ray irradiation of a human cancer cell line in vitro and in vivo. Int. J. Radiat. Oncol., Biol., Phys. 41: 669-674, 1998.

10. Frixen, U.H., Behrens, J., Sachs, M., et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol. 113: 173-185, 1991.

11. Gunji, N., Oda, T., Todoroki, T., et al. Pancreatic carcinoma - correlation between e-cadherin and alpha-catenin expression status and liver metastasis. Cancer 82: 1649-1656, 1998.

12. Isaacs, J.T. Molecular markers for prostate cancer metastasis. Developing diagnostic methods for predicting the aggressiveness of prostate cancer. Am. J. Pathol. 150: 1511-1521, 1997.

13. Jian, W.G., Darnton, S.J., Jenner, K., Billingham, L.J. and Matthews, H.R. Expression of E-cadherin in oesophageal carcinomas from the UK and China: disparities in prognostic significance. J. Clin. Pathol. 50: 640-644, 1997.

14. Krishnadath, K.K., Tilanus, H.W., van Blankenstein, M., et al. Reduced expression of the cadherin-catenin complex in oesophageal adenocarcinoma correlates with poor prognosis. J. Pathol. 182: 331-338, 1997.

15. Nakanishi, Y., Ochiai, A., Akimoto, S., et al. Expression of E-cadherin, alpha-catenin, beta-catenin and plakoglobin in esophageal carcinomas and its prognostic significance: immunohistochemical analysis of 96 lesions. Oncology 54: 158-165, 1997.

16. Pomp, J., Davelaar, J., Blom, J., et al. Radiotherapy for oesophagus carcinoma - the impact of p53 on treatment outcome. Radiother. Oncol. 46: 179-184, 1998.

17. Ruijter, E., Vandekaa, C., Aalders, T., et al. Heterogeneous expression of e-cadherin and p53 in prostate cancer - clinical implications. Modern Pathology 11: 276-281, 1998.

18. Tamura, S., Shiozaki, H., Miyata, M., et al. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br. J. Surg. 83: 1608-1614, 1996.

19. Umbas, R., Isaacs, W.B., Bringuier, P.P., et al. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res. 54: 3929-3933, 1994.

20. von Essen, C.F. Radiation enhancement of metastasis: a review. Clin. Exp. Metastasis 9: 77-104, 1991.

CHAPTER VI

P53 AND RADIOTHERAPY FOR OESOPHAGUS CARCINOMA: A COMPARISON BETWEEN 4 DIFFERENT ANTIBODIES.

J. Pomp,

J. Blom,

A.H. Zwinderman

C. van Krimpen

Reprinted from: Oncology Reports 7: 1075-1078, 2000
With permission from Professor D.A. Spandidos, editor

Abstract

Background and purpose; Wild type p53 protein plays an important role in the cellular response to ionising radiation and other DNA damaging agents and is mutated in many human tumours. Immunohistochemistry is a rapid method to detect elevated protein levels. The p53 status in a group of patients treated by radiotherapy exclusively for oesophagus carcinoma was examined and correlated with pre-treatment parameters and treatment outcome with regard to overall survival, local recurrence free survival and distant metastases free survival. In this paper, four different antibodies were used to evaluate their predictive power detecting p53 expression. *Material and Methods;* Immunostaining for p53 protein with 4 different antibodies was performed on paraffin-embedded specimens from 69 patients with adenocarcinoma and squamous cell carcinoma of the oesophagus. All patients were treated with radiotherapy exclusively, consisting of external irradiation combined with intraluminal brachytherapy. *Results;* Detection of p53 using the antibody (DO7) was significantly correlated with overall survival and distant metastases free survival. For local recurrence free survival no statistical significance was reached. The use of the other 3 antibodies all showed the same trend with regard to distant metastases, however statistical significance was never reached. The use of multiple antibodies did not increase the predictive value of DO7. Both for survival and metastases free survival the use of DO7 alone was sufficient as a significant prognostic factor. We conclude that in this series, only the use of DO7 was correlated with prognosis in oesophagus carcinoma treated by radiotherapy only and that addition of 3 antibodies did not improve the predictive power.

Introduction

P53 is a tumour suppressor gene, encoding for a nuclear phosphoprotein of 53 kilodalton (kDa). The protein product of this suppressor gene, wild type p53 protein, plays a role in the genomic stability, cell cycle control and DNA damage repair after treatment with ionising radiation and cytotoxic agents [14,15]. It is involved in the G_1 block after radiotherapy and regulates apoptotic response [3,11]. When mutated, p53 acts as an oncogene. As a consequence the G_1 cell cycle response after cytotoxic treatment is absent and apoptosis does not occur [13]. This could explain the increased radioresistance as observed mostly in vitro, [11,12,16] although not always confirmed by others [2,21,26]. Mutations of the p53 gene have been found in many tumours and amongst them the oesophagus [4,7,19]. Elevated protein level, which occurs in case of mutations, can be detected by immunohistochemistry (IHC). However IHC has a number of limitations and its sensitivity is estimated only to be 75% and the predictive value is only 63% [23]. We studied a group of patients with locally advanced oesophageal carcinoma, treated by radiotherapy exclusively, and determined the p53 protein expression by immunohistochemistry, using four different antibodies to investigate whether their combination would increase their predictive power with regard to overall, local recurrence and distant metastases free survival.

Material and methods

Patients

A group of 69 patients with locally advanced carcinoma of the oesophagus, treated by radiotherapy exclusively, was subject of investigation. No patients were known to have distant metastases at the time of diagnosis. From the tumours of these patients paraffin embedded archival material was available detect p53 expression by immunohistochemistry.

Antibodies.

P53 protein expression of the tumour specimens was detected with the aid of the immunoperoxidase method, according to the Streptavidin Biotin Complex (SAB Complex) method [17], using the following antibodies: Antibody BP53-12, mapping N-terminus aa. 20-25; Antibody clone PAb 1801, mapping aa. 32-79, and Ab-5 (Clone DO7), mapping aa 37-45. These antibodies (from Neomarkers, Fremont CA) react with wild type and mutant p53. The fourth was clone CM-1, a polyclonal rabbit antibody reacting with wild type and most mutant forms of p53 (Biogenx, San Ramon, CA). The size of the archival slides did not permit staining with all 4 antibodies for all cases: Sixty-nine slides were stained with the antibody 1801 while in 68 cases the p53 expression was determined by DO7, in 67 cases by PB 53-12 and 64 times by CM1.

Analysis

After staining, all slides were scored by two of the authors (CK and JP). Discrepancies were discussed till agreement was reached. Specimens were considered to be positive for p53 expression when at least 20% of the tumour cells were stained [5].

The concordance between the different antibodies used for testing p53 expression is described. Furthermore the correlation for each antibody separately, was studied between p53 expression and clinical parameters such as age, sex, weightloss, topography, length of tumour, histology, stage and N-classification. Overall survival (OS) and freedom from local recurrence as well as freedom from distant metastases was calculated for both p53- and p53+ tumours, using the Kaplan Meier method. The log-rank test was used to test the difference.

In multivariate Cox regression analysis, the effects of confounders (age, sex, weightloss, topography, length of tumour, histology, stage and N-classification and treatment dose) on survival was modelled in a forward stepwise manner. Thereafter, p53 status, as determined by each antibody separately, was forced into the model and tested for significance.

Results

Patients

At the time of evaluation with a follow up period of 2-9 years, 6 patients were still alive, and 63 had died of whom 19 with local recurrence, 15 with distant metastases only and 11 with both local and distant failure. Eighteen patients had died without tumour.

The 4 different antibodies used

In 61 of the total cases the expression of all 4 antibodies was determined; not all biopsies contained enough tumour to sufficiently estimate the staining of 4 antibodies. Eighteen times the slides were negative using 4 antibodies and 19 scored positive using 4 antibodies. In seven, eight and nine cases, 1,2 and 3 antibodies stained positive respectively. A good concordance ($p<0,0001$) existed between the expression of 4 antibodies tested; the correlation coefficients were 0.51 or more.

Correlation between pre-treatment parameters and p53 expression

No correlation between pre-treatment parameters like histology (adenocarcinoma, squamous cell carcinoma), length of tumour, topography, lymphnode status, stage, age and weightloss, for either antibody tested was found ($p>0.5$) except for the CM1; This antibody detected p53 expression significantly more in case of squamous cell carcinoma (18/25, P= 0.003).

Correlation between treatment outcome and p53 expression

Of the four antibodies tested, only the use DO7 in the detection of p53 expression correlated significantly with disease fee survival. (Table 1). This was due to a significant lower occurrence of distant metastases in the group of tumours that stained negatively for DO7. Time to local recurrence for DO7 negative tumours was longer than for D07 positive tumours, but this did not reach statistical significance. The other antibodies showed no correlation with either local or distant failure, although for 1801 a trend exists for p53 negative tumours having longer distant metastases free period.

Discussion

We tested whether immunohistochemically determined p53 expression could correlate with treatment outcome in a group of patients treated with radiotherapy for oesophagus carcinoma. In a previous study it was found that expression determined by the antibody DO7 strongly correlated with overall survival and distant metastases free survival [17]. To investigate whether the addition of more antibodies would increase the predictive value, four different antibodies were used. The other 3 antibodies, although showing the same trend, were not significantly correlated with treatment outcome. The addition of 3 extra antibodies to DO7 did not increase the predictive value ($p>0.58$).

P53 is an oncogene, mutated in many malignant tumours of different organs including the oesophagus. As it plays a role in the response to cytotoxic treatment, like ionising radiation, it might serve as a predictive factor for treatment outcome. Immunohistochemistry, revealing elevated levels of the p53 protein is a rapid way to determine overexpression of p53, which is assumed to correlate with mutations in the gene. However, immunohistochemical determination of the p53 status has a number of limitations [23]; wild type p53, for example, can show an increased expression [8] or p53 can be mutated without being detected by IHC [1]. The fixation of the archive material and the choice of antibodies, which is subject to variation plays a crucial role too [6,22]. Serbia [18] found no correlation with survival using D01 (67% stained positive), in a group of 204 patients having surgery for squamous cell carcinoma of the oesophagus.

Horne [9] compared the same four different antibodies for immunohistochemistry as we did, when looking at p53 as a prognostic factor in breast carcinoma; PAb1801, p53-BP-12, DO7 and CM1. It was concluded that Pab 1801 and DO7 were the most effective. However in their series the use of 1801 showed the strongest prediction for prognosis. Vet et al [24] tested the differences between IHC and single strand conformation polymorphism (SSCP) in bladdercarcinoma. They found a good concordance between SSCP and IHC. But it was noticed that p53 stability (leading to overexpression of p53 protein) was not merely triggered by mutation. This means that p53 staining can be positive while the protein is wild

Table 1

Median disease free survival, metastasis free period, and local recurrence free period (95%confidence interval) and the associated relative risk (95% confidence interval) for each of the 4 mutations.

		Negative	Positive	P#
Disease Free Survival (months):				
1801	median	11 (7-16)	7 (6-9)	0.29
	Relative Risk		1.32 (0.79-2.20)	
DO7	median	12 (6-19)	7 (2-12)	0.03
	Relative Risk		1.73 (1.04-2.89)	
PBP 53-12	median	9 (0-18)	8 (4-12)	0.43
	Relative Risk		1.23 (0.74-2.04)	
PCM1	median	8 (5-10)	10 (6-15)	0.79
	Relative Risk		0.93 (0.55-1.57)	
Metastasis Free Period (months):				
1801	median	>25*	14 (5-23)	0.61
	Relative Risk		1.24 (0.54-2.88)	
DO7	median	>25*	10 (7-14)	0.006
	Relative Risk		3.29 (1.35-8.00)	
BP 53-12	median	>14*	14 (7-21)	0.16
	Relative Risk		1.86 (0.78-4.42)	
PCM1	median	25 (0-63)	>14*	0.78
	Relative Risk		0.89 (0.38-2.06)	
Local Recurrence Free Period (months):				
1801	median	13 (6-19)	15 (7-22)	0.82
	Relative Risk		1.09 (0.52-2.30)	
DO7	median	14 (9-19)	10 (4-16)	0.23
	Relative Risk		1.55 (0.75-3.21)	
BP 53-12	median	13 (7-19)	15 (5-24)	0.79
	Relative Risk		1.10 (0.54-2.27)	
PCM1	median	13 (6-20)	15 (-)**	0.67
	Relative Risk		0.85 (0.39-1.84)	

* The median could not be estimated, it was larger than the largest time at which an event occurred.

** The 95% confidence interval could not be estimated.

type. Jacquemier et al. [10] tested 4 different antibodies in breast cancer (1801, 240, DO7 and DO1). While 40% of the tumours had at least 1 antibody positive, only 14% scored positive for all antibodies used. After performing SSCP on a selected group of specimens it was concluded that p53 overexpression was not representative for the number of cells with a mutation. Furthermore in their series of breastcarcinoma, p53 status, determined by either procedure was not correlated with disease free survival (DFS) or early recurrence. Xu et al. [25] using 3 different antibodies (Pab421, Pab240 and Pab1801) in head and neck cancer found large differences in immunopositivity (varying from 32 to 53 %). In contrast with Vet et al, in their series, SSCP results correlated poorly with p53 expression as determined by IHC. As an explanation for false positive and false negative IHC findings, nonsense and frameshift mutations are put forward. Schneider et al [20] pointed out that the discrepancy between IHC and SSCP varies amongst tumours.

We conclude from our series, that the use of DO7 for the detection of p53 expression, served as a good indicator for prognosis in a group of patients with locally advanced oesophagus carcinoma. It was strongly correlated with the occurrence of distant metastases. The additional use of 3 other antibodies did not increase its predictive value with regard to prognosis.

Acknowledgements:

The generous help of Dr. H.F. Eggink, pathologist in Leeuwarden for the provision of all the tissue blocks is gratefully acknowledged. We would like to thank Dr. P. Spoelstra, gastro-enterologist, Leeuwarden for the carefully performed endoscopies before treatment and during follow up and the co-operation during the Intraluminal Brachytherapy. The data management carried out by Ms. K.J. v.d. Elsaker is highly appreciated.
This work was supported by a grant from the scientific projects committee of the Reinier de Graaf Group, Delft.

Literature

1. Bodner, S.M., Minna, J.D., Jensen, S.M., et al. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. Oncogene 7: 743-749, 1992.

2. Brachman, D.G., Beckett, M., Graves, D., Haraf, D., Vokes, E. and Weichselbaum, R.R. p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines [see comments]. Cancer Res. 53: 3667-3669, 1993.

3. Clarke, A.R., Purdie, C.A., Harrison, D.J., et al. Thymocyte apoptosis induced by p53-dependent and independent pathways [see comments]. Nature 362: 849-852, 1993.

4. Coia, L.R. and Sauter, E.R. Esophageal cancer. Curr. Probl. Cancer 18: 189-247, 1994.

5. Cordon Cardo, C., Dalbagni, G., Saez, G.T., et al. p53 mutations in human bladder cancer: genotypic versus phenotypic patterns. Int. J. Cancer 56: 347-353, 1994.

6. Fisher, C.J., Gillett, C.E., Vojtesek, B., Barnes, D.M. and Millis, R.R. Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. Br. J. Cancer 69: 26-31, 1994.

7. Galiana, C., Lozano, J.C., Bancel, B., Nakazawa, H. and Yamasaki, H. High frequency of Ki-ras amplification and p53 gene mutations in adenocarcinomas of the human esophagus. Molecular. Carcinogenesis 14: 286-293, 1995.

8. Hall, P.A. and Lane, D.P. p53 in tumour pathology: can we trust immunohistochemistry? Revisited! [editorial] [see comments]. J. Pathol. 172: 1-4, 1994.

9. Horne, G.M., Anderson, J.J., Tiniakos, D.G., et al. p53 protein as a prognostic indicator in breast carcinoma: a comparison of four antibodies for immunohistochemistry. Br. J. Cancer 73: 29-35, 1996.

10. Jacquemier, J., Moles, J.P., Penault Llorca, F., et al. p53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. Br. J. Cancer 69: 846-852, 1994.

11. Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311, 1991.

12. Lee, J.M. and Bernstein, A. p53 mutations increase resistance to ionizing radiation. Proc. Natl. Acad. Sci. USA 90: 5742-5746, 1993.

13. Lowe, S.W., Ruley, H.E., Jacks, T. and Housman, D.E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957-967, 1993.

14. Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes [see comments]. Nature 362: 847-849, 1993.

15. Maity, A., McKenna, W.G. and Muschel, R.J. The molecular basis for cell cycle delays following ionizing radiation: areview. Radiother. Oncol. 31: 1-13, 1994.

16. McIlwrath, A.J., Vasey, P.A., Ross, G.M. and Brown, R. Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. Cancer Res. 54: 3718-3722, 1994.

17. Pomp, J., Davelaar, J., Blom, J., et al. Radiotherapy for oesophagus carcinoma - the impact of p53 on treatment outcome. Radiother. Oncol. 46: 179-184, 1998.

18. Sarbia, M., Porschen, R., Borchard, F., Horstmann, O., Willers, R. and Gabbert, H.E. p53 protein expression and prognosis in squamous cell carcinoma of theesophagus. Cancer 74: 2218-2223, 1994.

19. Sauter, E.R., Keller, S.M. and Erner, S.M. p53 correlates with improved survival in patients with esophagealadenocarcinoma. J. Surg. Oncol. 58: 269-273, 1995.

20. Schneider, J., Rubio, M.P., Rodriguez Escudero, F.J., Seizinger, B.R. and Castresana, J.S. Identification of p53 mutations by means of single strand conformation polymorphism analysis in gynaecological tumours: comparison with the results of immunohistochemistry. Eur. J. Cancer 30A: 504-508, 1994.

21. Slichenmyer, W.J., Nelson, W.G., Slebos, R.J. and Kastan, M.B. Loss of a p53-associated G_1 checkpoint does not decrease cell survival following DNA damage. Cancer Res. 53 :4164-4168, 1993.

22. Tormanen, U., Eerola, A.K., Rainio, P., et al. Enhanced apoptosis predicts shortened survival in non-small cell lung carcinoma. Cancer Res.. 55: 5595-5602, 1995.

23. Velculescu, V.E. and Eldeiry, W.S. Biological and clinical importance of the p53 tumor suppressor gene. Clin..Chemistry. 42: 858-868, 1996.

24. Vet, J.A., Bringuier, P.P., Schaafsma, H.E., Witjes, J.A., Debruyne, F.M. and Schalken, J.A. Comparison of P53 protein overexpression with P53 mutation in bladder cancer: clinical and biologic aspects. Lab. Invest. 73: 837-843, 1995.

25. Xu, L., Chen, Y.T., Huvos, A.G., et al. Overexpression of p53 protein in squamous cell carcinomas of head and neck without apparent gene mutations. Diagn. Mol. Pathol. 3: 83-92, 1994.

26. Zaffaroni, N., Benini, E., Gornati, D., Bearzatto, A. and Silvestrini, R. Lack of a correlation between p53 protein expression and radiation response in human tumor primary cultures. Stem.Cells Dayt. 13: 77-85, 1995.

CHAPTER VII

CELL DENSITY DEPENDENT PLATING EFFICIENCY AFFECTS OUTCOME AND INTERPRETATION OF COLONY FORMING ASSAYS.

J. Pomp

J.L. Wike

I.J.M. Ouwerkerk

C. Hoogstraten

J. Davelaar

P.I. Schrier

J.W.H. Leer

H.D. Thames

W.A. Brock

Reprinted from: Radiotherapy and Oncology 40: 121-125, 1996
With permission from Elsevier Science

Abstract

Background and purpose: The usefulness of colony forming assays (CFA) has been established for almost 40 years [8]. Although time consuming and not successful for all cell lines, it is generally considered to be the gold standard of assays for testing the sensitivity of cell lines to ionizing radiation or other cytotoxic agents in vitro. We recently found for several cell lines that the plating efficiencies of both control and irradiated cells is dependent upon the density of cells seeded for colony formation; that is, increasing cell inoculum levels resulted in a non-linear relationship with colony formation, even at relatively low colony numbers. *Material and methods*: All data from a human melanoma cell line, transfected with c-myc or N-ras as well as from normal human diploid fibroblasts were taken to see how this phenomenon influenced outcome and interpretation of clonogenic assays. Survival was recalculated using all data, or only data with a linear relationship between inoculum level and colony formation. *Results*: It is found that when data with a non-linear relationship between inoculum level and colony formation are included, survival can be underestimated due to inhibition of colony formation in treated cultures. *Conclusion*: For validity, colony forming assays must be standardized to assure a constant relationship between the cell density and colony forming efficiency. This usually requires a much lower density of colonies than has been typically published for many cell survival studies.

Introduction

Colony forming assays (CFA) are used to determine the fraction of cells that survive after in vitro cytotoxic treatments (eg. irradiation). Cell survival is defined as the ratio of the plating efficiencies (PE), (i.e. the percentage of cells seeded that grow into macro colonies) between treated and untreated cells. When determining PE, the assumption is commonly made that PE is independent of the number of cells seeded into culture, at least over a wide range of cell numbers. For statistical reasons, cells are generally seeded to obtain a relatively constant number of surviving macro-colonies in each culture dish, typically ranging from 25 to 200 colonies. To accomplish this, the number of cells seeded must increase with the treatment dose. This approach is valid, of course, only if colony formation is independent of cell seeding density. While investigating the role of oncogene transfection on radiation sensitivity of a human melanoma cell line (IGR39) (Pomp et al, submitted for publication) [7], we found that the plating efficiency of the parental line and all of its derivatives was not constant with respect to the number of seeded cells. This was found to be true in both untreated and radiation treated cultures. Higher cell inocula resulted in the formation of fewer that expected colonies. This cell density dependent PE was not restricted to malignant or normal cells and was also independent of the presence or status of transfected oncogenes. The objective of this study was to examine for the occurrence of a non-linear relationship between colony formation and seeding density in cell lines of various origins and to determine the effects on cell survival measurements.

Material and Methods

Cell lines

The human melanoma and fibroblast cell lines were kept at 37°C in a 5% CO_2-flushed incubator in DMEM supplemented with penicillin/streptomycin, 1.5% glutamine (melanoma cell lines) and 8% heat-inactivated fetal calf serum (melanoma cell lines) or 15% fetal bovine medium serum (human fibroblasts).

The human melanoma cell line IGR39D derived from IGR39 [1] and its transfectants are described elsewhere [6,9]. Briefly, this melanoma cell line was transfected with the oncogenes c-myc (IGRmyc) and mutated N-ras (61-Arginine)(IGRras). A cell line transfected with the neomycinphosphotransferase resistance gene, conferring resistance to G418, was used as a control (IGRneo).

Normal skin fibroblast cultures, all in early passage, were derived from a group of patients that are part of an ongoing study, determining the relationship between the survival at 2 Gy (SF_2) and late reaction after radiotherapy for breast cancer. The data from two strains with different radiosensitivities were used to calculate SF_2. These were designated

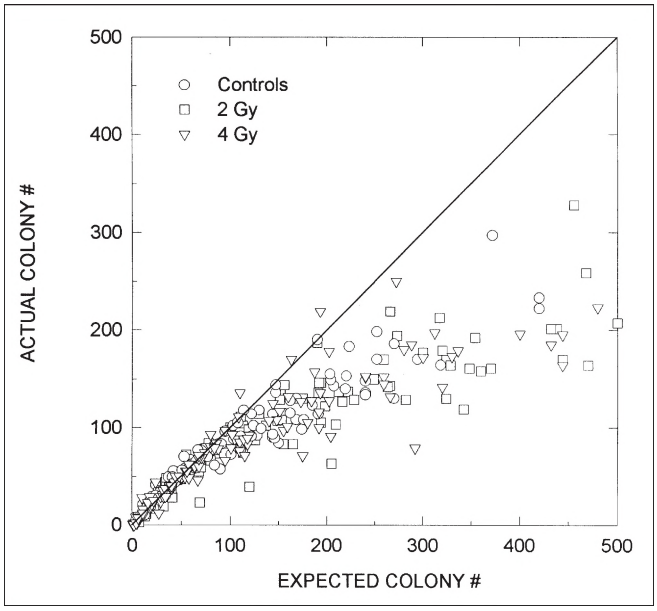


Figure 1
Density dependent plating efficiency in human skin fibroblasts. Colony numbers exceeding 70 colonies per dish are no longer linear with the number of cells seeded.

BAR1 (SF_2 = 0.24), and S33 (SF_2 = 0.45). The data from all of these cell lines were used to compose Figure 1.

Plating efficiency

Plating efficiencies for the different cell lines were assessed. For human melanoma cell lines, plating efficiencies ranged from 20-50% and for human fibroblasts from 3-60%. Exponentially growing or confluent cultures were made into single cell suspensions, checked for multiplicity, seeded into culture dishes (60 mm for melanoma cells and 100 mm for fibroblasts), and incubated at 37° with 5% CO_2 in air. Melanoma colonies were fixed with 96% ethanol and stained with 0.4% Coomassie Brilliant Blue. Fibroblast colonies were fixed with 70% ethanol and stained with 0.5% crystal violet. Melanoma colonies were fixed after two weeks in culture and fibroblast colonies were fixed at variable times, depending upon their growth rate. Colonies containing 50 or more cells were scored as survivors.

Irradiation protocol

Cell suspensions from three human melanoma cell lines cells (IGRneo, IGRmyc and IGRras) were prepared from exponentially growing or confluent cultures and plated in 60 mm dishes containing 5 ml medium. Three cell inoculum levels, each in triplicate, were plated for each radiation dose delivered. Cells were allowed to attach for four hours and were then irradiated with 0 to 4.0 Gy at room temperature using a 5 MeV or 6 MeV accelerator. After irradiation the cultures were returned to the incubator for colony formation. For

Table 1

Three different methods for calculating survival from clonogenic assays.

Method 1	Survival based on pooled plating efficiency data from all inoculum levels.
Method 2	Survival based on lowest inoculum level per dose (linear data).
Method 3	Survival based on equal inoculum levels at all doses.

human fibroblasts, a slightly different protocol was used. Early passage fibroblasts, both in confluent and exponential growth, were irradiated with 0-3.0 Gy and then seeded in 100 mm dishes, using 3-5 different cell inoculum levels, each in duplicate. After 2-3 weeks of incubation, fibroblast colonies were fixed, stained and counted.

Three different methods of selecting data for survival analysis were evaluated, as described in Table 1.

Results

Plating efficiency

The data from the 22 early passage human fibroblast strains were pooled in order to examine the relationship between the number of cells seeded and plating efficiency. The results for control and after three different radiation doses are shown in Figure 1. As can be seen, there appears to be a linear relationship between the number of cells inoculated and colony number until a level of approximately 70 colonies per 100 mm dish is reached. But as the number of cells per dish increases, the total number of colonies does not increase at the same rate. This change at 70 colonies/dish occurs for both irradiated and unirradiated cells. In the human melanoma cell lines, the relationship between cells inoculated and colony number becomes non-linear after 40-50 colonies per dish (data not shown). On the other hand, a CHO cell line (AA8) and an adenocarcinoma cell line (Clone A) do not show this phenomenon. In these cases, colony number and inoculum size have a linear relationship no matter how many cells are inoculated. (Figure 2)

To determine whether this apparent inhibition of colony formation was due to the surface area available per cell seeded, the PE data from figure 1 was plotted against the culture surface available for each cell (Figure 3). The plating efficiency increases with increasing area, but it reaches a plateau after 150 mm^2/cell, which is only 52 cells in a 100 mm culture dish. Similar results were found for the melanoma cell lines IGRneo, IGRmyc and IGRras (data not shown).

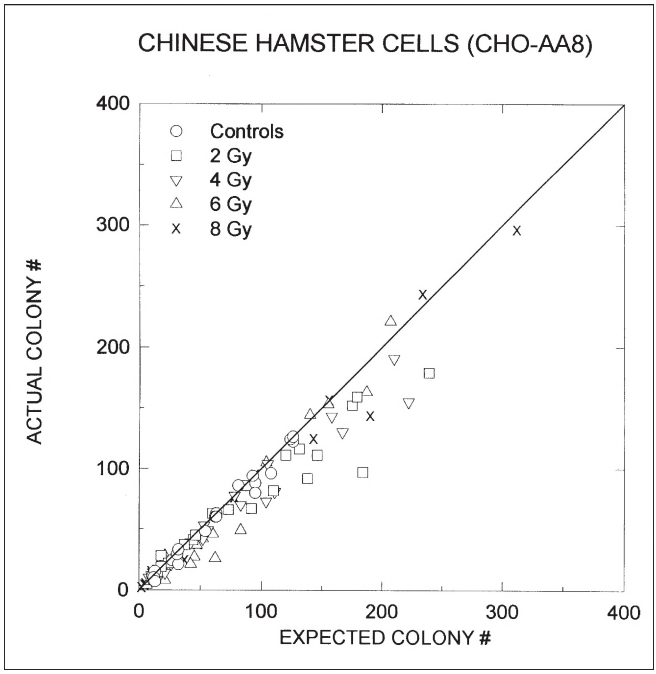


Figure 2
The Chinese hamster cell line, CHO-AA8, has a constant plating efficiency, that is independent of the number of cells inoculated.

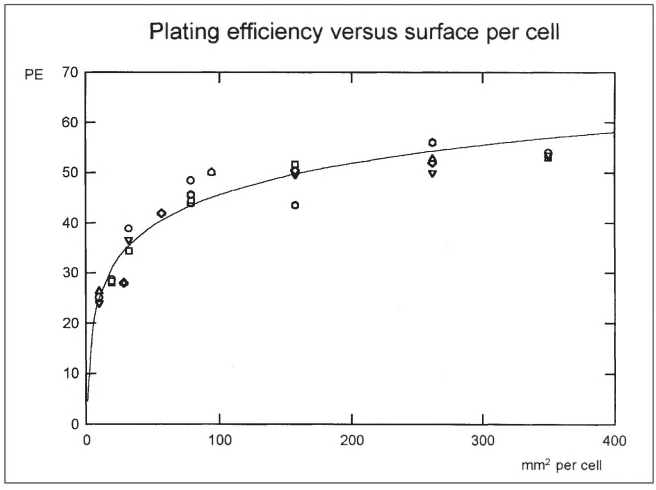


Figure 3
The relationship between culture surface area available per cell and plating efficiency is shown. The data were taken from the unirradiated cells in Table 1.

Radiation experiments

Since measurements of cell sensitivity to cytotoxic treatments are based upon accurate PE measurements, PE artifacts caused by different cell seeding densities must be properly controlled. The neo-, c-myc and N-ras transfectants of the human melanoma cell line IGR39D and normal human skin fibroblasts were irradiated with graded doses from 0-4.0

Gy. Plates were seeded with 3 different inoculum levels at all radiation doses and the plating efficiencies were determined. Since different plating efficiencies were observed at different inoculum levels after the same dose of irradiation, selecting data for survival curves is problematical. Therefore, survival curves were calculated by three different methods, but all according to the equation: $^{e}\log S = -\alpha D - \beta D^2$. First, survival was calculated by pooling all the data obtained from the experiments (Table 1, method 1 and Table 2,1). Second, survival was calculated while only taking into account plating efficiencies that show a linear relationship with the numbers of cells seeded (i.e. low inoculum levels, Table 1, method 2 and Table 2, 2). The survival curves as based on these data are shown in Figure 4, where S33 (a human fibroblast line) is taken as an example. It can be seen that data from the lowest inoculum had the highest levels calculated survival, while plating efficiencies taken from the highest inoculum level lead to lower estimates of survival. When using pooled data or high inoculum levels, the lowest survival was calculated, probably due to an underestimation of plating efficiency. From this we conclude that only data derived from the range where a linear relationship between plating efficiency and colony formation exists leads to the best estimation of survival.

In the third method, survival was calculated by comparing plating efficiencies from an equal level of inoculum for untreated and treated cells. As cell killing is higher after larger doses of irradiation, these data can only be obtained for low radiation doses; otherwise the

***Table* 2**

SF_2 results calculated from each of the 3 methods listed in Table 1.

	SF_2		
	1	2	3
IGRneo	0.57	0.64	0.56-0.81
IGRmyc	0.52	0.48	0.46-0.63
IGRras	0.38	0.39	0.43-0.57
BAR1	0.16	0.24	n.a.[a]
S33	0.4	0.45	n.a.

Results of SF_2 as calculated from the 3 methods listed in Table 1.

Method 1 Survival based on pooled plating efficiency data from all inoculum levels.

Method 2 Survival based on lowest inoculum level per dose (linear data).

Method 3 Survival based on equal inoculum levels at all doses.

[a] Not available

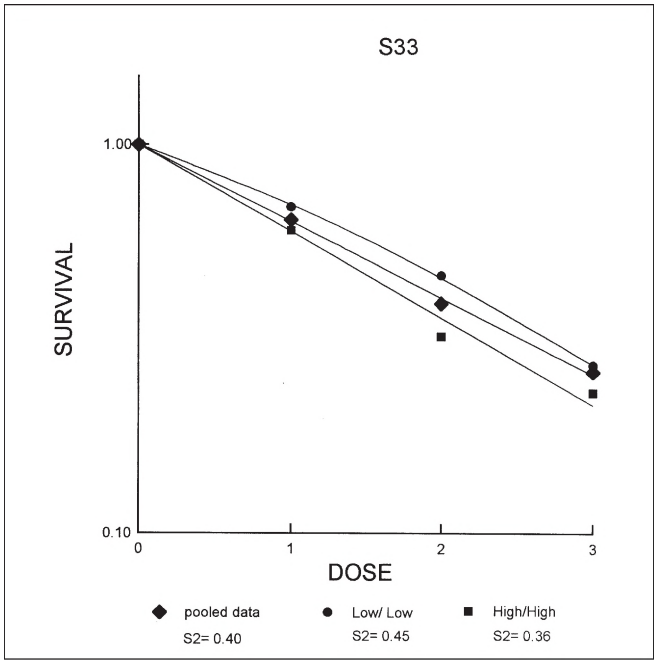


Figure 4
Survival curves of fibroblast line S33 , after survival was calculated from pooled data, low inoculum levels or high inoculum levels.

number of colonies after treatment will be too low to be statistically significant. The data were not available for BAR1 and S33. The purpose of this approach was to determine if it is the total numbers of cells seeded that contribute to plating efficiency reduction or the absolute number of surviving cells. Results for the cell lines IGRneo, IGRmyc and IGRras are listed in Table 2, Row 3, and it can be seen that different surviving fractions are calculated, depending on the inoculum level chosen. If PE depends upon the total number of seeded cells, survival would be constant. From this we conclude that it is the absolute number of surviving cells and not the total number of seeded cells that contribute to the cell density dependent plating efficiency.

We investigated whether the growth rate of the cells at the moment of inoculation could influence this phenomenon. As it occurs both in the case when cells were taken from exponential growth or confluence, the plating efficiency was dependent on seeding density.

Discussion

The validity of clonogenic assays is dependent upon plating efficiency being independent of the different numbers of cells inoculated in each experiment. However, we have found for a number of cell lines, that plating efficiency is dependent upon the cell seeding density within the critical range of cell numbers used in survival assays. This appears to be

true for human cell lines of various origins, including malignant melanoma, with and without transfected oncogenes, and for normal skin fibroblasts. At relatively high inoculum levels, plating efficiency was found to be highly dependent on the cell concentration placed into culture. Since we do not know the mechanism responsible for these observations, it is necessary to include controls to correct for this problem [3,4]. This is accomplished by using five inoculum levels for each radiation dose and then use only those data in which a linear relationship between the numbers of cells inoculated and colonies formed is apparent. This same phenomenon was also observed in rodent cell lines (3T3 fibroblasts, data not shown) and in both treated and untreated cells. By testing three different methods of selecting data from clonogenic assays, we illustrate both the importance of examining each cell line for this problem and also methods to use controls to be certain the survival data are valid. That is, simply, to use only those data which have been shown to have a linear relationship between cell number and PE.

By taking into account only equal inoculum levels, we concluded that it is the absolute number of surviving cells that contribute to the inhibition of colony formation. Therefore, the bending point, i.e. the number of colonies that is no longer linearly related to the number of seeded cells, is not always the same and must be determined experimentally. When radiation experiments are performed, the exact number of survivors is not known beforehand, so in case of cell density dependent plating efficiency, it is obligatory to validate plating efficiency measurements by inoculating a range of different cell numbers.

The discovery of a non-linearity of colony formation is not new. Eliason et al [2] reported that primary cultures from human tumor biopsies, exhibit plating efficiencies that are dependent on cell seeding densities. In 1983, Meyskens et al [5] published that murine melanoma cells have a non-linear relationship between plating efficiency and cell numbers. It appeared that only colonies smaller than 70 μm exhibit a linear relationship. These authors also showed that survival (after treatment with DTIC) can be erroneously higher than controls at low toxicity level.

Conclusion

Our observations point out the importance of standardizing clonogenic assays when testing for cell sensitivity to ionizing radiation or chemotherapeutic drugs in vitro. Cell density dependent plating efficiency differences are common, unpredictable, and can lead to an incorrect interpretation of survival data.

Acknowledgements.

The authors wish to thank A. Boon, dept of clinical oncology, Leiden, for the assistance with the radiation experiments and Mrs. M. Winterwerp-Thoms, dept. of radiotherapy, Delft, for the careful typing of the data.

References

1. Aubert, C., Rouge, F., and Galindo, J.R. Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation in vitro. J. Nat. Cancer Inst. 64: 1029-1040, 1980.

2. Eliason, J.F., Aapro, M.S., Decrey, D., and Brink-Petersen, M. Non-linearity of colony formation by human tumour cells from biopsy samples. Br. J. Cancer 52: 311-318, 1985.

3. Geara, F.B., Peters, L.J., Ang, K.K., Wike, J.L., and Brock, W.A. Radiosensitivity measurement of keratinocytes and fibroblasts from radiotherapy patients. Int. J. Radiat. Oncol. Biol. Phys. 24: 287-293, 1992.

4. Geara, F.B., Peters, L.J., Ang, K.K., Wike, J.L., Sivon, S.S., Guttenberger, R., Malaise, E.P., and Brock, W.A. Intrinsic radiosensitivity of normal human fibroblasts and lymphocytesafter high- and low-dose-rate irradiation. Cancer Res. 52: 6348-6352, 1992.

5. Meyskens, F.L., Jr., Thomson, S.P., Hickie, R.A., and Sipes, N.J. Potential biological explanation of stimulation of colony growth in semi-solid agar by cytotoxic agents. Br. J. Cancer 48: 863-868, 1983.

6. Peltenburg, L.T.C., Steegenga, W.T., Krüse, K.M., and Schrier, P.I. c-Myc-induced Natural Killer cell sensitivity of human melanoma cells is reversed by HLA-B27 transfection. Eur. J. Immunol. 22: 2737-2740, 1992.

7. Pomp, J., Ouwerkerk, I.J.M., Hermans, J., Wondergem, J., Cornelisse, C.J., Leer, J.W.H., and Schrier, P.I. The influence of oncogenes N-ras and c-myc on the radiation sensitivity of human melanoma cell lines. Radiation Research 1996.

8. Puck, T.T. and Marcus, P.I. Action of X-rays on mammalian cells. J. Exper. Med. 103: 653-666, 1956.

9. Van Elsas, A., Van Deursen, E., Wielders, R., Van den Berg-Bakker, C.A.M., and Schrier, P.I. Ras oncogene activation does not induce sensitivity to natural killer cell-mediated lysis in human melanoma. J. Invest. Dermatol. 103: 117S-121S, 1994.

CHAPTER VIII

SUMMARY AND DISCUSSION / SAMENVATTING EN DISCUSSIE

Summary and discussion

The theme of this thesis explores the contribution of oncogenes to radiation response of malignant cells. Proto-oncogenes play a role in DNA damage recognition, the regulation of checkpoints, and are often involved in DNA damage repair [13,25,26,47], for example after ionising radiation [3,5,29] and cell cycle control [14]. They do not contribute to malignant development under normal conditions. In the case of alteration by mutation or overexpression by gene amplification, proto-oncogenes are called oncogenes. Oncogenes deregulate basic molecular pathways in the cell and can cause cancer. As proto-oncogenes are involved in cell cycle regulation and DNA damage repair after radiotherapy, it is possible that oncogenes can modify the radiation response, thereby affecting the treatment outcome. Therefore it is of interest to understand the effects of oncogenes on radiosensitivity.

Firstly, we investigated whether oncogene expression in malignant cells alters radiosensitivity and secondly which mechanisms play a role. Both *in vitro* data using a human melanoma cell line, and clinical data collected from patients with oesophagus carcinoma treated by high dose radiotherapy provided material for this thesis.

Chapter 1 starts with a short introduction to basic radiobiology. This is indispensable to interpret the *in vitro* data from the clonogenic and cell cycle assays. The function of oncogenes in normal and tumour cells is explained and how they may play a role in the radiation response and cell cycle regulation. The last part of the chapter summarises the layout of the thesis and which questions served as a guidance to fulfil this study.

In **Chapter 2** we describe how oncogene transfection affected the radiation response of a human melanoma cell line. A human melanoma cell line (IGR39D) with normal expression of *MYC* and without mutated *NRAS* was transfected with either oncogene or both. *NRAS* contained a mutation in codon 61 (NRAS 61-arginine); *MYC* was brought to overexpression by transfection. We showed that the survival after single dose irradiation was decreased after transfection with *MYC* or *NRAS*. When both oncogenes were transfected into the parental cell line, survival was reduced only in the first part of the survival curve (up to 4 Gy), after which it resembled the parental line.

At first glance this result is in contrast with early literature describing an increased radiation resistance after transfection with the oncogene *H-ras* in rodent cell lines. The most likely explanation is that our melanoma cell line differed from the cell lines that were used for the original papers, correlating oncogene expression with radiation response. The cell lines used in the early nineties were rat embryonic fibroblasts. These non-malignant cell lines probably had no oncogene activation, a wild type p53 status and last but not least, expressed proto-oncogenes necessary for embryonic development.

p53 is involved both in G_1 and G_2 delay and mutated p53 may overrule the effect of

other oncogenes that are involved in radiation response. Although no consensus exists whether p53 by itself can be held responsible for altered radiosensitivity (except in lymphocytes systems) it may influence the expression and function of other oncogenes [12]. The melanoma cell line we used was of human origin and malignant. We regarded this as a better reflection of the clinical situation of human tumours with activation of oncogenes, which may be involved in the radiation response. The p53 status as detected by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) showed that this gene was truncated in the parental cell line we used. Therefore this cell line should probably be considered p53 null.

Cellular response to ionising radiation is complex and involves multiple pathways. An oncogene-related effect might be unnoticed if an alternative pathway annuls its effect, so that in the cell line used, this oncogene status does not affect the phenotypic features of radiosensitivity. Furthermore, an effect on radiation response can only become clear if the whole pathway downstream this oncogene is intact.

In summary, the influence of one single oncogene on radiation response will depend on the status of the total complex of pathways involved in DNA damage recognition, repair and cell cycle regulation.

In the literature on rodent material, oncogene-related increased radioresistance was accompanied by a longer G_2 delay after ionising radiation. This phenomenon was used as an explanation for increased survival after radiotherapy. In **Chapter 3** we describe cell cycle effects after radiotherapy of the parental and the *RAS*-transfected melanoma cell lines. Cells were labelled with propidium iodide to measure the total DNA content, and with BrdUrd to determine the progression of cells through S phase. By looking at the DNA content at time intervals after radiation, the accumulation of cells in G_2 was established. It was found that the G_2 delay in the *NRAS*-containing cell line was prolonged. This is in part consistent with the literature. The data published earlier with rat embryonal fibroblasts also showed that cells transfected with *H-ras* had a longer radiation induced G_2 delay than the parental cells [37]. However, in contrast with the conclusion from these authors, we could not confirm that prolonged G_2 delay was a feature of radioresistance.

Beside the human origin and being derived from a malignant tumour, our cell lines differed from the rodent cell lines in p53 status. P53 is responsible for the G_1 arrest after radiation and more recently it has been suggested that p53 may also be involved in G_2 delay [43] either directly or via p21/Cip1 [18]. Neither the parental melanoma cell line, nor the oncogene transfected daughter cell lines showed any G_1 delay, due to mutations in the p53 status.

The G_2 checkpoint delays mitosis to check for DNA damage and allow for repair. DNA damage repair needs to be completed before mitosis starts in order to guarantee the generation of two normal daughter cells. Prolongation of G_2 is therefore a manifestation of DNA

damage and occurs after radiotherapy and other genotoxic insults. Altered regulation of cyclines and cycline dependent kinases are required to cause the G_2 delay.

Attempts have been made to correlate the length of the G_2 delay with radiation response and intrinsic radiosensitivity. If a correlation between radiosensitivity and the length of G_2 delay exists, it could be used as a predictive factor for the cellular response to radiotherapy. This topic is controversial; cells of ataxia telangiectasia (AT) patients, which are very radiosensitive, show prolonged G_2 delay [2,10,31,32] suggesting a correlation between the duration of G_2 delay and radiosensitivity, while in rodent systems, prolonged G_2 delay after oncogene transfection was used as an explanation for oncogene induced radioresistance. For both phenomena different explanations can be put forward. First there is the importance of an intact G_2 checkpoint. When the G_2 checkpoint is normal, prolonged G_2 delay is an expression of increased DNA damage, which should correlate with radiosensitivity. On the other hand can prolonged G_2 delay allow for increased DNA repair resulting in increased survival (radioresistance). Permanent arrest in the G_2 phase is one form of clonogenic death. Malfunctioning of the checkpoint, leading to an inadequate G_2 delay, would leave DNA damage unrepaired, resulting in increased kill. This so-called mitotic catastrophe is another form of clonogenic death. So, without knowledge about the intactness of the G_2 checkpoint and other cell cycle regulating mechanisms, the relation ship between G_2 delay and radiation response cannot properly be interpreted. The fact that G_2 delay is dose dependent (and corresponds with reduced survival), also suggests that the duration of the G_2 is a reflection of DNA damage and more likely to be a feature of radiosensitivity.

By labelling cells with BrdUrd just before irradiation, progression through the S phase could be followed. After 5 Gy we found no significant S phase delay. It appeared, however, that the S phase shortly after irradiation was slightly reduced, but the total duration of the S-phase was not impaired, as expected from previous studies [27]. There was no difference in S phase duration between the parental and the *NRAS* transfected cell line.

In conclusion, based on the literature and our own experiments, we found there is no basis to assume a direct correlation between G_2 delay and radiosensitivity. Therefore we conclude that the effect of ionising radiation on G_2 delay is not correlated with radioresistance in general but depends on the system under investigation. G_2 delay cannot be used as a predictor for radiosensitivity.

The role of p53 in the radiation response after radiotherapy is explored in **Chapter 4**. As mentioned earlier, wildtype p53 plays a role in cell division, cell cycle regulation and the integrity of the genome. It is important for the G_1 arrest and involved in apoptosis induction after radiotherapy. The reduction in apoptosis when p53 is mutated has been put forward as an explanation for radioresistance. However, whether apoptosis is a sign of radiosensitivity is controversial [6,48]. Apoptosis may have a more important impact on fractionated radiotherapy than on single dose radiation [42]. Furthermore, p53 is not the only determinant for

apoptosis. p53 independent stress responses, regulating cell growth, should not be overlooked. Apoptosis and G_1 arrest can also occur without activation of p53 or WAF/CIP1 expression [20]. The p53 response differs in different normal tissues and does therefore not always have the same effect on radiation response [36]. Some papers have correlated mutated p53 with an increased radioresistance and an increased resistance to cytotoxic therapy [17,30,33-35] while other paper could not confirm this [4,6,9,16,28,49].

In this clinical study, we questioned whether p53 status played a role in the local control of oesophagus carcinoma treated by high dose radiotherapy only. A retrospective study is described which was performed on material of patients with oesophagus carcinoma who had been treated by radiotherapy exclusively. We investigated the role of p53 on local control and the impact on survival. Biopsy material was available of 69 patients prospectively followed in a non-randomised study. Paraffin embedded tissue blocks were used to determine the p53 status by immunohistochemistry. Expression of p53 was correlated with overall survival, local recurrence free survival and the occurrence of distant metastases. The overall survival was significant higher for patients with negative p53 staining (wild type p53). Local disease free survival did not correlate with p53 status. Based on these data we could not conclude that p53 status is associated with altered radiation response. The metastases-free survival was worse for mutated p53 tumours.

As discussed previously, the status of one single oncogene can not determine radiosensitivity. Preferably the presence of all oncogenes that are related to survival after ionising radiation should be considered; this still remains difficult at the present time. Another explanation why no direct correlation between local control (a measure for radiosensitivity) was found, may be the staining method of p53. Not all mutations lead to detectable overexpression by immunohistochemistry. Furthermore the influence of mutated p53 on radiosensitivity is tumour type dependent and different mutations may have different impact on radiosensitivity [11].

Radiotherapy is aimed to provide local-regional control. Local control is important and reduces the risk of distant metastases [40,41,44]. If local control cannot be obtained, the risk of distant metastases is increased. Oncogenes are involved in the radiation response. Since radiosensitivity may be affected by oncogenes, we hypothesise that oncogene expression may also have an impact on the occurrence of distant metastases. This may either be by a direct effect on metastatic behaviour, or an indirect effect via mechanisms involved in the radiation response and altered local tumour control.

When we discovered that p53 expression correlated with increased distant metastases in a group of patients with oesophagus carcinoma, we also studied e-cadherin expression, another oncogene involved in the development of distant metastases. The results are given in **chapter 5**. In this chapter a correlation between e-cadherin and a range of pre-treatment parameters, including p53 was looked at. No relation could be found between de different

parameters studied. With regard to treatment outcome we found a relation between e-cadherin expression pattern and the occurrence of distant metastases. When an aberrant staining pattern for e-cadherin was found, the risk of distant metastases was reduced.

In general, it is believed that normal e-cadherin expression prevents the occurrence of distant metastases, which is in contrast with our findings. However, our data are in agreement with a paper on pancreatic tumours where intact e-cadherin was associated with an increased risk on distant metastases. The explanation given was that the cells needed close contact, provided by normal e-cadherin expression, in order to establish metastases [23].

The rationale to connect radiation response, oncogene expression and metastases is based on literature from the seventies, suggesting that radiotherapy can alter metastatic behaviour of tumours [46] and more recent literature, showing that radiation can upregulate the expression of e-cadherin [1,19].

A possible correlation between e-cadherin and p53 was first suggested in a study with p53 deficient mice, which developed skin tumours with altered expression of e-cadherin [8] and in a clinical study correlating p53 and e-cadherin in a series of breast carcinomas [7]. The explanation is that a relationship between p53 and e-cadherin could involve β-catenin. β-catenin promotes the accumulation of transcriptionally active p53 by interference with degradation of p53. This is probably an indirect effect since a decrease in e-cadherin enhances the release of β-catenin into the cytosol and nucleus and this may trigger p53 stabilisation [15].

The influence of p53 on the occurrence of distant metastases does not necessarily involve catenins and cadherin only. Many more metastasis-related genes are under the influence of p53 like matrix metalloproteinase (MMP), cathepsin D, thrombospondin-1 [45]. Whether these oncogenes have an impact on distant metastases in tumours treated by radiotherapy is not known.

Last but not least, we must take into account all the limitations of the methods used. In **chapter 6**, four different antibodies were used to evaluate their predictive power detecting p53 expression. Detection of p53 using the antibody DO7 correlated significantly with overall survival and distant metastases free survival. For local recurrence free survival no level of significance was reached. The use of the other 3 antibodies all showed the same trend with regard to distant metastases, however no significance was ever reached. The use of multiple antibodies did not increase the predictive value of DO7 [24].

The pitfalls of clonogenic assays are described in **chapter 7**. We found that for a number of cell lines the density of the seeded cells had an impact on plating efficiency; when too many cells were seeded, plating efficiency was inhibited leading to an underestimation of the plating efficiency. This could occur for untreated as well as for treated dishes. It had hardly been noticed in literature [21,38] that the survival fraction could be underestimated

due to this, leading to underestimation of SF_2.

We therefore recommend that the phenomenon of cell density dependent plating efficiency should always be tested before results from such assays are interpreted.

Conclusions

After one decade and many papers on the correlation between oncogene expression and radiation response, no clear answer has been given whether this is relevant for radiotherapy. The participation of oncogenes in the response after ionising radiation is clearly complex. When an oncogene has a role in DNA damage recognition or repair, or cell cycle regulation, an influence on radiation response can be anticipated. Today the knowledge of the expression status of one single oncogene is not considered enough to predict outcome after radiotherapy, as the contribution of a single oncogene in the response to radiotherapy can never be seen as a sole factor. [5]. We conclude that due to the complexity of interactions of many mechanisms involved in radiation response, the determination of a limited number of oncogenes can not be used to predict the outcome of radiotherapy

In vitro experiments have established that oncogenes can influence radiosensitivity but this depends on the cell line under study. The best example so far is p53. Whether p53 leads to apoptosis or G_1 arrest after ionising radiation depends on the tissue that is looked at [11,22,39]. Consequently, mutations in p53 cannot predict eventual alterations in radiosensitivity.

With regard to cell cycle control and ionising radiation, the correlation between G_2 delay and radiation response remains unclear, as the correct interpretation of the role of the G_2 delay is not exactly defined yet.

Another interesting link with radiation response is the occurrence of distant metastases. From clinical research it is known that local control reduces the development of distant metastases. Different mechanisms may relate radiation responses and metastatic pattern. First a common factor influencing both radiosensitivity and the risk on distant metastases may exist. Second, the influence of radiotherapy by itself on the occurrence of distant metastases (eventually via oncogene pathways) deserves further study.

Samenvatting en discussie

De effecten van oncogenen op de respons van maligne cellen na bestraling zijn onderwerp van studie voor dit proefschrift. Proto-oncogenen spelen een rol bij de herkenning van DNA schade en de regulatie van controle punten tussen de verschillende fasen van de cel cyclus (checkpoints). Zij zijn vaak betrokken bij het herstel van DNA schade [13,25,26,47], zoals bijvoorbeeld na bestraling [3,5,29] en de gepaard gaande effecten op de celcyclus [14]. Onder normale omstandigheden dragen proto-oncogenen niet bij aan maligne ontaarding van cellen. In geval van verandering door mutatie of overexpressie door genamplificatie, worden proto-oncogenen oncogenen genoemd. Oncogenen kunnen allerlei enzymatische routes in de cel verstoren en op die manier mede verantwoordelijk zijn voor het ontstaan van kanker. Aangezien proto-oncogenen betrokken zijn bij de regulatie van de cel cyclus en reparatie van DNA schade na radiotherapie, is het mogelijk dat oncogenen de reactie na radiotherapie veranderen. Daarom is het nodig meer inzicht te krijgen in de effecten van oncogenen op de stralen gevoeligheid van cellen.

Ten eerste werd onderzocht of oncogen expressie in kwaadaardige cellen de stralen gevoeligheid van deze cellen verandert en vervolgens welke mechanismen hierbij een rol spelen. Zowel *in vitro* data van een humane melanoom cellijn en klinische data, verzameld van patiënten met oesophaguscarcinoom, behandeld met radiotherapie, leverden het materiaal voor dit proefschrift.

Hoofdstuk 1 start met een korte introductie in de radiobiologie. Dit is onmisbaar om *in vitro* data van clonogene assays en mechanismen van cel cyclus regulatie te begrijpen. De functie van proto-oncogenen wordt besproken en hoe zij betrokken kunnen zijn bij de reactie op bestraling en de regulatie van de celcyclus. Het laatste deel van dit hoofdstuk vat het onderzoek samen en welke vragen wij ons hierbij gesteld hebben.

In **Hoofdstuk 2** wordt beschreven hoe oncogen-transfectie de radiatie respons van een humane melanoom cellijn beïnvloedt. Een humane melanoom cellijn (IGR39D) met normale expressie van *MYC* en zonder gemuteerd *NRAS* werd getransfecteerd met elk oncogen afzonderlijk of beide. Het *NRAS* oncogen bevat een mutatie in codon 61 (*NRAS* 61-arginine); *MYC* werd tot overexpressie gebracht middels transfectie. Aangetoond werd dat de overleving na eenmalige bestraling was verminderd na transfectie met *MYC* of *NRAS*. Wanneer beide oncogenen werden getransfecteerd in de parentale cellijn was de overleving weliswaar verminderd maar alleen in het eerste deel van de overlevingscurve tot 4 Gy. Daarna was de overleving hetzelfde die van de parentale lijn.

Op het eerste gezicht lijken deze resultaten in contrast met de vroege literatuur welke een verhoogde radioresistentie beschreef van knaagdier cellijnen die getransfecteerd waren het oncogen *H-ras*. De meest waarschijnlijke verklaring hiervoor is dat onze melanoom cel-

lijn verschilt van de cellijnen die gebruikt werden voor de oorspronkelijke literatuur, waarin oncogen expressie in verband gebracht werd met de reactie op bestraling. De cellijnen die oorspronkelijk werden gebruikt waren rat embryonale fibroblasten. Deze niet maligne cellijnen hadden waarschijnlijk geen oncogen activatie, bevatten een wild type p53 en brachten proto-oncogenen tot expressie nodig zijn voor embryonale ontwikkeling.
p53 is betrokken zowel bij de G_1 als de G_2 delay en gemuteerd p53 zou het effect kunnen overschaduwen van andere oncogenen welke betrokken zijn bij de respons op radiotherapie. Er bestaat geen consensus of gemuteerd p53 zelf verantwoordelijk kan zijn voor veranderderde stralen gevoeligheid (behalve in lymfocyten systemen), maar het zou wel de expressie en functie van andere oncogenen kunnen beïnvloeden [12]. De melanoom cellijn door ons gebruikt, was van humane origine en maligne. Wij beschouwden dit als een betere weergave van de klinische situatie van humane tumoren met activatie van oncogenen welke betrokken kunnen zijn bij de reactie op radiotherapie. Polymerase chain reaction-single strand conformation polymorphisme (PCR-SSCP) toonde aan dat p53 in onze parentale cellijn was getrunceerd. Daarom beschouwden wij onze cellijn als p53 nul type.

De cellulaire respons op bestraling bestaat uit meerdere stappen. Een oncogen gerelateerd effect zou ongemerkt kunnen blijven als een alternatieve route dit effect opheft, waardoor op het fenotype van stralengevoeligheid niet verandert. Een eventueel effect van een oncogen op response na radiotherapie kan alleen worden opgepikt als de alle volgende stappen in de reactie op radiotherapie intact zijn. De invloed van een enkel oncogen op de radiatie respons is afhankelijk van het totale complex van reacties betrokken bij herkenning en reparatie van DNA schade en de regulatie van de celcyclus.

In de literatuur over cel lijnen van knaagdieren ging oncogen gerelateerde radioresistentie samen met een toegenomen verlenging van de G_2 fase (G_2 delay). Dit fenomeen werd gebruikt als een verklaring voor verhoogde overleving na radiotherapie. In **Hoofdstuk 3** worden de celcyclus effecten van de parentale en de *RAS* getransfecteerde melanoom cellijnen na radiotherapie beschreven. Cellen werden gelabeld met propidium iodide om de totale DNA inhoud te meten en met BrdUrd om de progressie van de cellen door de S fase te volgen. Door de DNA inhoud vast te stellen op verschillende tijdsstippen na bestraling werd de stapeling van cellen in de G_2 gemeten. De G_2 delay in de *RAS* bevattende cellijn was verlengd. Dit is deels in overeenstemming met de literatuur. De eerder gepubliceerde data met rat embryonale fibroblasten toonde ook dat cellen die getransfecteerd waren met *H-ras* een langere G_2 delay hadden na bestraling dan de parentale cellijnen [37]. In contrast met de conclusie van deze auteurs, konden wij echter de verlengde G_2 delay niet in verband brengen met radioresistentie.

Naast het feit dat onze cellen van humane origine en afkomstig van een maligne tumor waren, verschilden onze cellijnen met die uit de literatuur, in p53 status. P53 is een proto-oncogen, verantwoordelijk voor de G_1 arrest na bestraling en is ook in verband gebracht met

de G_2 delay [43] hetzij via directe beïnvloeding of indirect via p21/Cip1 [18]. Noch de parentale melanoom cellijn, noch de oncogen getransfecteerde dochter cellijn tonen enige G_1 delay hetgeen wordt toegeschreven aan mutaties in p53.

Het G_2 checkpoint vertraagt de mitose zodat DNA schade kan worden opgespoord en gerepareerd. De reparatie van schade aan het DNA moet voltooid zijn voor de mitose begint om het ontstaan van twee normale dochterstellen te waarborgen. Verlenging van de G_2 fase is daarom een uiting van DNA schade en treedt op na radiotherapie en andere genotoxische schade. Veranderende regulatie van cyclines en cyclinen afhankelijke kinases zijn verantwoordelijk voor deze G_2 delay.

Er zijn pogingen ondernomen om de duur van de G_2 delay te correleren met de reactie op radiotherapie en de intrinsieke radiosensitiviteit. Indien er een correlatie tussen de stralengevoeligheid en de lengte van de G_2 delay bestaat, dan zou deze gebruikt kunnen worden om de cellulaire respons op radiotherapie te voorspellen. Dit onderwerp is controversieel; cellen van ataxia telangiectasia (AT) patiënten die erg gevoelig zijn voor bestraling, vertonen een verlengde G_2 delay [2,10,31,32], hetgeen een correlatie suggereert tussen de duur van de G_2 delay en stralengevoeligheid, terwijl in knaagdier cellijnen, verlengde G_2 delay juist werd gebruikt als een verklaring voor oncogen geïnduceerde radioresistentie. Voor beide effecten bestaan verschillende verklaringen. Allereerst is het van belang dat het G_2 checkpoint intact is. Wanneer het G_2 checkpoint normaal functioneert, betekent verlengde G_2 fase vaststelling en reparatie van meer DNA schade, hetgeen past bij stralengevoeligheid. Een verlengde G_2 delay kan ook gelegenheid bieden voor betere reparatie van DNA schade, met als gevolg hogere overleving (stralenresistentie). Permanente arrest in de G_2 fase is één van de vormen van clonogene dood. Wanneer het checkpoint niet of onvoldoende functioneert, hetgeen een niet optimaal G_2 delay tot gevolg heeft, kan de mitose ingezet worden met DNA schade welke niet voldoende gerepareerd is, resulterend in toegenomen cel dood. Deze zogenoemde mitotische catastrofe is ook een vorm clonogene dood. Het is onmogelijk om zonder kennis over het al dan niet goed functioneren van het G_2 checkpoint en andere celcyclus regulerende mechanismen, een uitspraak te doen over de relatie tussen G_2 delay en radiatie respons. Het feit dat de G_2 delay dosis afhankelijk is (en correspondeert met verminderde overleving) suggereert dat de duur van de G_2 delay een weergave is van de mate van DNA schade en daarmee hoogstwaarschijnlijk een uiting van stralengevoeligheid.

Door cellen juist voor de bestraling met BrdUrd te labelen kan de voortgang door de S fase worden gevolgd. Na 5 Gy vonden we geen significante S fase delay. Het leek er echter op dat de S fase kort na de bestraling enigszins vertraagd was maar de totale duur van de S fase was niet veranderd zoals uit voorgaande studies werd verwacht. Er was geen verschil in S fase duur tussen de parentale en de *NRAS* getransfecteerde cellijn.

In conclusie, gebaseerd op de literatuur en onze eigen experimenten vonden we geen onderbouwing voor een directe correlatie tussen de G_2 delay en radiosensitiviteit. Daarom

concluderen we dat het effect van straling op G_2 delay niet gecorreleerd is met radioresistentie in het algemeen, maar afhangt van het systeem dat onderzocht wordt. G_2 delay kan derhalve niet gebruikt worden als een voorspellende factor voor radiosensitiviteit.

De rol van p53 in de reactie op radiotherapie wordt behandeld in **Hoofdstuk 4.** Zoals eerder vermeld speelt wild type p53 een rol in de celdeling, celcyclus regulatie en het intact houden van het genetisch materiaal. p53 is essentieel voor de G_1 arrest en is betrokken bij de inductie van apoptose na radiotherapie. Een verminderde apoptose in geval van mutatie in het p53 gen, is aangevoerd als een verklaring voor verminderde gevoeligheid voor bestraling. Of apoptose zelf een teken is van radiosensitiviteit blijft controversieel [6,48]. Mogelijk dat apoptose van meer belang is bij gefractioneerde radiotherapie dan na één enkele dosis [42]. p53 is niet de enige factor betrokken bij inductie van apoptose; Apoptose en G_1 arrest kunnen ook optreden zonder activatie van p53 of WAF/CIP1 expressie [20]. De p53 respons varieert tussen verschillende normale weefsels en dit verklaart waarom p53 de reactie op radiotherapie verschillend kan beïnvloeden [36]. In sommige artikelen is een gemuteerde p53 status gecorreleerd met toegenomen radioresistentie en een toegenomen resistentie voor cytotoxische therapie [17,30,33-35], terwijl dit in andere publicaties niet het geval is [4,6,9,16,28,49].

In deze klinische studie werd onderzocht of de p53 status een rol speelt bij de locale controle van middels hoge dosis radiotherapie behandeld oesophagus carcinoom. Een retrospectief onderzoek werd uitgevoerd op materiaal van patiënten met een oesophagus carcinoom behandeld met radiotherapie. We keken naar de invloed van p53 op locale tumor controle en de overleving. Biopsie materiaal was beschikbaar van 69 patiënten welke prospectief vervolgd waren in een niet gerandomiseerde studie. Paraffine weefselblokjes werden gebruikt om de p53 status door middel van immunohistochemie te bepalen. Expressie van p53 werd gecorreleerd met de overall survival, local recurrence free survival en het optreden van afstandsmetastasen. De overall survival was significant hoger voor patiënten met een negatieve p53 kleuring (wild type p53). Locale ziektevrije overleving was niet gecorreleerd aan de p53 status. Gebaseerd op deze data kon niet geconcludeerd worden dat de p53 status geassocieerd is met veranderde radiatie respons. De metastasevrije overleving was slechter voor p53 gemuteerde tumoren.

Opnieuw blijkt dat het niet mogelijk is om met de status van één enkele oncogen de radiosensitiviteit te voorspellen. Het liefst zouden alle oncogenen betrokken bij de reactie op radiotherapie in beschouwing moeten worden genomen. Dit is momenteel nog erg moeilijk. Een andere mogelijkheid, waarom geen directe correlatie tussen locale controle (een maat voor radiosensitiviteit) en de p53 status werd gevonden, kan te maken hebben met de methode van p53 bepaling. Niet alle mutaties worden opgepikt door immunohistochemie. Verder kan de invloed van gemuteerd p53 op stralengevoeligheid afhankelijk zijn van het tumortype en verschillende p53 mutaties kunnen verschillende impact op de radiosensi-

tiviteit hebben [11].

Radiotherapie is gericht op het verkrijgen van locoregionale controle. Locale controle is belangrijk en reduceert de kans op het ontstaan van afstandsmetastasen [40,41,44]. Oncogenen zijn betrokken bij de respons op radiotherapie. Aangezien radiosensitiviteit zou kunnen worden beïnvloed door oncogenen, vroegen wij ons af of oncogen expressie ook een invloed kan hebben op het ontstaan van afstandsmetastasen. Dit effect kan zowel direct zijn op het metastatische gedrag van tumorcellen of indirect via mechanismen die de stralengevoeligheid en locale controle beïnvloeden.

Na de bevinding dat p53 expressie correleerde met verhoogde afstandsmetastasen in patiënten met oesophaguscarcinoom, werd in deze groep de expressie van e-cadherin, een oncogen betrokken bij de ontwikkeling van afstandsmetastasen, bestudeerd. De resultaten zijn weergegeven in **Hoofdstuk 5.** In dit hoofdstuk wordt de relatie tussen e-cadherin en andere voor de behandeling aanwezige parameters en de uitkomst wat betreft overleving en lokaal recidief onderzocht. We vonden een relatie tussen e-cadherin expressie en het optreden van afstandsmetastasen. Wanneer e-cadherin abnormaal tot expressie kwam waren er minder afstandsmetastasen.

In het algemeen wordt aangenomen dat normale e-cadherin expressie het optreden van afstandsmetastasen voorkomt. Dit is in tegenspraak met onze bevindingen. Echter onze data zijn wel in overeenstemming met een artikel over pancreas tumoren waarin intact e-cadherin juist geassocieerd is met verhoogd kans op afstandsmetastasen. De verklaring hiervoor zou kunnen zijn dat cellen weliswaar niet te sterk aan elkaar vast moeten zitten om los te komen, maar normale e-cadherin expressie nodig hebben om afstandsmetastasen te vormen.

Het idee om reactie op bestraling, oncogen expressie en afstandsmetastasen met elkaar in verband te brengen, is gebaseerd op literatuur van de vroege 70 jaren waarin gesuggereerd wordt dat radiotherapie het metastatisch karakter van tumoren kan beïnvloeden [46] en meer recente literatuur welke aantoont dat bestraling de expressie van e-cadherin kan aanschakelen [1,19].

Een mogelijke correlatie tussen e-cadherin en p53 werd gesuggereerd in een studie met p53 deficiënte muizen die huidtumoren ontwikkelden met veranderde e-cadherin expressie [8] en in een klinische studie waarin p53 en e-cadherin gecorreleerd bleken te zijn in een serie mammacarcinomen [7]. Via b-catenin kan een mogelijke een relatie tussen p53 en e-cadherin worden verklaard. Vermindering van e-cadherin bevordert het vrijkomen van b-catenin in het cytosol en de nucleus en b-catenin bevordert de ophoping van transcriptioneel actief p53 door afbraak van de p53 te verhinderen [15].

De invloed van p53 op het ontstaan van afstandsmetastasen hoeft niet alleen gerelateerd te zijn aan catenins en e-cadherin. Er zijn meer afstandsmetastasen gerelateerde genen welke onder de invloed van p53 staan, zoals matrix metalloproteinase (MMP), cathepsin D,

thrombospondin-1 [45]. Of deze oncogenen van belang zijn bij het optreden van metastasen van tumoren die behandeld zijn met radiotherapie is nog niet bekend.

Van belang voor onderzoek zijn ook de beperkingen van de methoden die gebruikt worden. In **Hoofdstuk 6** werden 4 verschillende antibodies getest om de voorspellende waarde van p53 expressie te bepalen. De bepaling van p53 met gebruikmaking van het antibody DO7 correleerde significant met overall survival en optreden van afstandsmetastase vrije overleving. Voor het optreden van lokaal recidief werd geen significantie bereikt. Het gebruik van 3 andere antibodies toonde allemaal dezelfde trend met betrekking tot afstandsmetastasen maar geen van alle werd significant. Het gebruik van meerdere antibodies verhoogde niet de voorspellende waarde van DO7 [24].

De valkuilen van clonogene assays worden beschreven in **Hoofdstuk 7**. Wij vonden voor een aantal cellijnen dat de dichtheid van de uitgezaaide cellen een invloed had op de plating efficiency; wanneer te veel cellen worden uitgezaaid wordt de plating efficiency verminderd, hetgeen kan leiden tot onderschatting van de plating efficiency. Dit kan zowel optreden voor behandelde als niet behandelde schaaltjes. Dit is nauwelijks beschreven in de literatuur [21,38]. Op deze manier kan de overlevingsfractie worden onderschat en daarmee de SF2. Wij raden daarom aan dat het fenomeen van cel dichtheid afhankelijke plating efficiency altijd wordt getest voordat de resultaten van de assays worden geïnterpreteerd

Conclusies

Na 10 jaar en vele artikelen over de correlatie tussen oncogen expressie en de reactie op bestraling is er geen duidelijk antwoord op de vraag of dit relevant is voor de klinische radiotherapie. De betrokkenheid van oncogenen in de reactie na bestraling is heel complex. Indien een oncogen een rol heeft in de herkenning van DNA schade, reparatie van DNA schade of de celcyclus regulatie, lijkt een invloed op radiatie respons aannemelijk. Tegenwoordig is de kennis van de expressie van een enkel oncogen niet genoeg om de uitkomst na radiotherapie te voorspellen omdat een enkel oncogen deel uitmaakt van een complex geheel [5]. We concluderen dat ten gevolge van de vele interacties en mechanismen betrokken bij de reactie op radiotherapie, het niet mogelijk is om met een beperkt aantal oncogenen te voorspellen hoe de uitkomst na radiotherapie zal zijn.

In vitro experimenten hebben aangetoond dat oncogenen de response op radiotherapie kunnen beïnvloeden maar de manier waarop hangt af van de cellijn. Het beste voorbeeld tot nu toe is het oncogen p53. Of p53 leidt tot apoptose of G_1 arrest na bestraling hangt af van het weefsel dat bekeken wordt [11,22,39]. Daarom kan een mutatie in p53 niet een

eventuele verandering in radiosensitiviteit voorspellen.

Met betrekking tot regulatie van de celcyclus na bestraling blijft de exacte relatie tussen G_2 delay en radiatie respons onduidelijk omdat de correcte interpretatie van de rol van G_2 delay niet gedefinieerd is.

Een ander interessant verband met radiatie respons is het optreden van afstandsmetastasen. Uit klinisch onderzoek is bekend dat locale controle de ontwikkeling van afstandsmetastase vermindert. Verschillende mechanismen kunnen mogelijk een verband leggen tussen radiatie respons en metastaseringspatroon. Allereerst zou er een gemeenschappelijke factor kunnen bestaan die zowel de radiosensitiviteit als de kans op afstandsmetastasen beïnvloedt. Ten tweede dient de invloed van radiotherapie zelf op het optreden van afstandsmetastasen verder te worden onderzocht; het is niet ondenkbaar dat hierbij oncogenen betrokken zijn.

Literature

1. Akimoto, T., Mitsuhashi, N., Saito, Y., Ebara, T. and Niibe, H. Effect of radiation on the expression of e-cadherin and alpha-catenin and invasive capacity in human lung cancer cell line in vitro. Int. J. Radiat. Oncol., Biol., Phys. 41: 1171-1176, 1998.

2. Beamish, H. and Lavin, M.F. Radiosensitivity in ataxia-telangiectasia - anomalies in radiation-induced cell cycle delay. Int. J. Radiat. Biol. 65: 175-184, 1994.

3. Bernhard, E.J., Maity, A., Muschel, R.J. and McKenna, W.G. Effects of ionizing radiation on cell cycle progression. A review. Radiat. Environ. Biophys. 34: 79-83, 1995.

4. Brachman, D.G., Beckett, M., Graves, D., Haraf, D., Vokes, E. and Weichselbaum, R.R. p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines [see comments]. Cancer Res. 53: 3667-3669, 1993.

5. Bristow, R.G., Benchimol, S. and Hill, R.P. The p53 gene as a modifier of intrinsic radiosensitivity -Implications for radiotherapy [Review]. Radiother. Oncol. 40: 197-223, 1996.

6. Brown, J.M. and Wouters, B.G. Apoptosis, p53, and tumor cell sensitivity to anticancer agents [review]. Cancer Research 59: 1391-1399, 1999.

7. Bukholm, I.K., Nesland, J.M., Karesen, R., Jacobsen, U. and Borresen Dale, A.L. Expression of E-cadherin and its relation to the p53 protein status in human breast carcinomas. Virchows Arch. 431: 317-321, 1997.

8. Cano, A., Gamallo, C., Kemp, C.J., et al. Expression pattern of the cell adhesion molecules. E-cadherin, P-cadherin and alpha 6 beta 4 intergrin is altered in pre-malignant skin tumors of p53-deficient mice. Int. J. Cancer 65: 254-262, 1996.

9. Ceraline, J., Deplanque, G., Duclos, B., et al. Relationships between p53 induction, cell cycle arrest and survival of normal human fibroblasts following dna damage [french]. Bulletin du Cancer 84: 1007-1016, 1997.

10. Chen, P., Farrell, A., Hobson, K., Girjes, A. and Lavin, M. Comparative study of radiation-induced G2 phase delay and chromatid damagein families with ataxia-telangiectasia. Cancer Genet.Cytogenet. 76: 43-46, 1994.

11. Chiarugi, V., Magnelli, L. and Cinelli, M. Role of p53 mutations in the radiosensitivity status of tumor cells. Tumori. 84: 517-520, 1998.

12. Chiarugi, V., Magnelli, L., Cinelli, M., Turchetti, A. and Ruggiero, M. Dominant oncogenes, tumor suppressors, and radiosensitivity. Cell Mol. Biol. Res. 41: 161-166, 1995.

13. Chung, D.C. and Rustgi, A.K. DNA mismatch repair and cancer. Gastroenterology 109: 1685-1699, 1995.

14. Coleman, W.B. and Tsongalis, G.J. Multiple mechanisms account for genomic instability and molecular mutation in neoplastic transformation. Clin. Chem. 41: 644-657, 1995.

15. Damalas, A., Ben-Ze'ev, A., Simcha, I., et al. Excess beta-catenin promotes accumulation of transcriptionally active p53. EMBO Journal 18: 3054-3063, 1999.

16. Danielsen, T., Smith-Sorensen, B., Gronlund, H.A., Hvidsten, M., Borresen-Dale, A.L. and Rofstad, E.K. No association between radiosensitivity and tp53 status, G_1 arrest or protein levels of p53, *MYC*, *RAS* or *RAF* in human melanoma lines. Int. J. Radiat. Biol. 75: 1149-1160, 1999.

17. Dittmer, D., Pati, S., Zambetti, G., et al. Gain of function mutations in p53. Nat. Genet. 4: 42-46, 1993.

18. Dulic, V., Stein, G.H., Far, D.F. and Reed, S.I. Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G_2/M-phase transition. Mol. Cell Biol. 18: 546-557, 1998.

19. Ebara, T., Mitsuhashi, N., Saito, Y., Akimoto, T. and Niibe, H. Change in e-cadherin expression after x-ray irradiation of a human cancer cell line in vitro and in vivo. Int. J. Radiat. Oncol., Biol., Phys. 41: 669-674, 1998.

20. El Deiry, W.S., Harper, J.W., O'Connor, P.M., et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 54: 1169-1174, 1994.

21. Eliason, J.F., Aapro, M.S., Decrey, D. and Brink-Petersen, M. Non-linearity of colony formation by human tumour cells from biopsy samples. Br. J. Cancer 52: 311-318, 1985.

22. Flatt, P.M., Price, J.O., Shaw, A. and Pietenpol, J.A. Differential cell cycle checkpoint response in normal human keratinocytes and fibroblasts. Cell Growth & Differentiation 9: 535-543, 1998.

23. Gunji, N., Oda, T., Todoroki, T., et al. Pancreatic carcinoma - correlation between e-cadherin and alpha-catenin expression status and liver metastasis. Cancer 82: 1649-1656, 1998.

24. Hall, P.A. and Lane, D.P. p53 in tumour pathology: can we trust immunohistochemistry? Revisited! [editorial] [see comments]. J. Pathol. 172: 1-4, 1994.

25. Hanawalt, P.C. Role of transcription-coupled DNA repair in susceptibility to environmental carcinogenesis. Environ.Health Perspect. 104 Suppl 3: 547-551, 1996.

26. Harris, C.C. p53 tumor suppressor gene: at the crossroads of molecular carcinogenesis, molecular epidemiology, and cancer risk assessment. Environ. Health Perspect. 104 Suppl 3: 435-439, 1996.

27. Higashikubo, R., Ragouzis, M. and Roti Roti, J.L. Flow cytometric BrdUrd-pulse-chase study of X-ray-induced alterations in cell cycle progression. Cell Prolif. 29: 43-57, 1996.

28. Jung, M., Notario, V. and Dritschilo, A. Mutations in the p53 gene in radiation-sensitive and -resistant human squamous carcinoma cells. Cancer Res. 52: 6390-6393, 1992.

29. Kastan, M.B. Hot papers - oncology - participation of p53 protein in the cellular response to DNA damage by Kastan, M.B., Onyekwere, O., Ssidransky, D., et al. Scientist. 7: 16, 1993.

30. Kuo, M.L., Chou, Y.W., Chau, Y.P. and Huang, T.S. Resistance to apoptosis induced by alkylating agents in v-Ha-ras-transformed cells due to defect in p53 function. Mol. Carcinog. 18: 221-231, 1997.

31. Lavin, M.F., Bennett, I., Ramsay, J., et al. Identification of a potentially radiosensitive subgroup among patients withbreast cancer [see comments]. J. Natl. Cancer Inst. 86: 1627-1634, 1994.

32. Lavin, M.F., Le Poidevin, P. and Bates, P. Enhanced levels of radiation-induced G2 phase delay in ataxia telangiectasia heterozygotes. Cancer Genet. Cytogenet. 60: 183-187, 1992.

33. Lee, J.M. and Bernstein, A. p53 mutations increase resistance to ionizing radiation. Proc. Natl. Acad. Sci.USA 90: 5742-5746, 1993.

34. Lowe, S.W., Bodis, S., McClatchey, A., et al. p53 status and the efficacy of cancer therapy in vivo. Science 266: 807-810, 1994.

35. Lowe, S.W., Ruley, H.E., Jacks, T. and Housman, D.E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957-967, 1993.

36. MacCallum, D.E., Hupp, T.R., Midgley, C.A., et al. The p53 response to ionising radiation in adult and developing murine tissues. Oncogene 13: 2575-2587, 1996.

37. McKenna, W.G., Iliakis, G., Weiss, M.C., Bernhard, E.J. and Muschel, R.J. Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc. Radiat. Res. 125: 283-287, 1991.

38. Meyskens, F.L., Jr., Thomson, S.P., Hickie, R.A. and Sipes, N.J. Potential biological explanation of stimulation of colony growth in semi-solid agar by cytotoxic agents.

Br.J.Cancer 48: 863-868, 1983.

39. Midgley, C.A., Owens, B., Briscoe, C.V., Thomas, D.B., Lane, D.P. and Hall, P.A. Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo. J.Cell Sci. 108: 1843-1848, 1995.

40. Overgaard, M., Hansen, P.S., Overgaard, J., et al. Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. Danish Breast Cancer Cooperative Group 82b Trial [see comments]. N. Engl. J. Med. 337: 949-955, 1997.

41. Overgaard, M., Jensen, M.B., Overgaard, J., et al. Postoperative radiotherapy in high-risk postmenopausal breast-cancer patients given adjuvant tamoxifen: Danish Breast Cancer Cooperative Group DBCG 82c randomised trial. Lancet 353: 1641-1648, 1999.

42. Rupnow, B.A., Murtha, A.D., Alarcon, R.M., Giaccia, A.J. and Knox, S.J. Direct evidence that apoptosis enhances tumor responses to fractionated radiotherapy. Cancer Res. 58: 1779-1784, 1998.

43. Stewart, N., Hicks, G.G., Paraskevas, F. and Mowat, M. Evidence for a second cell cycle block at G2/M by p53. Oncogene 10: 109-115, 1995.

44. Suit, H.D. Local control and patient survival. Int. J. Radiat. Oncol. Biol. Phys. 23: 653-660, 1992.

45. Sun, Y., Wicha, M. and Leopold, W.R. Regulation of metastasis-related gene expression by p53: a potential clinical implication. Mol. Carcinog. 24: 25-28, 1999.

46. von Essen, C.F. Radiation enhancement of metastasis: a review. Clin. Exp. Metastasis 9: 77-104, 1991.

47. Williams, J.K. Principles of genetics and cancer. Semin. Oncol. Nurs. 13: 68-73, 1997.

48. Wouters, B.G., Giaccia, A.J., Denko, N.C. and Brown, J.M. Loss of p21(waf1/cip1) sensitizes tumors to radiation by an apoptosis-independent mechanism. Cancer Research 57: 4703-4706, 1997.

49. Zaffaroni, N., Benini, E., Gornati, D., Bearzatto, A. and Silvestrini, R. Lack of a correlation between p53 protein expression and radiation response in human tumor primary cultures. Stem.Cells Dayt. 13: 77-85, 1995.

DANKWOORD / ACKNOWLEDGEMENTS

Veel mensen hebben mij geholpen en bijgestaan tijdens de periode waarin dit proefschrift tot stand kwam. Niet alleen heb ik al die jaren veel hulp bij de uitvoering gekregen maar ook geweldige mentale support. Deze lijst zal helaas zeker niet volledig zijn. Daarom dank ik iedereen voor alle hulp in de afgelopen jaren. Een paar mensen wil ik graag nog apart noemen.

Ilse Ouwerkerk. Ilse, dank zij jou ben ik wegwijs gemaakt en heb ik geleerd wat werken in het lab is. Jij hebt enorm veel voor me gedaan, niet alleen als onderzoeker maar ook als mens. Jouw vriendschap is me in jaren ook erg dierbaar geworden. Dank je voor al je steun.

Koos Zwinderman. Koos, jij als statisticus, rechtlijnig denkend, maar met gevoel voor artsen. Mij sturend naar duidelijke vraagstelling en heldere formulering bleek een grote bijdrage voor de totstandkoming van het tweede deel van mijn proefschrift

Peter Schrier. Peter, je bent met mij gestart in de wereld van de moleculaire biologie die veel geduld vereist. Dat zal ik niet vergeten.

Adrian Begg. Adrian, your discussions and suggestions were always very stimulating. Ik am very happy with your help which contributed very much to the origin of this thesis.

Connie Hoogstraten. Connie, jij stuurde me door de wirwar van DNA hoeveelheden. Dank je voor het werk in de cel cyclus en de begeleiding op de FACS

Jaap Davelaar. Jaap, je stond altijd klaar en het was zo gezellig op je kamer terwijl jij de figuren maakte.

Kees van Krimpen. Kees, ik heb veel van je geleerd op het gebied van de PA. Ik hoop dat we ook na dit tijdperk nog veel onderzoek samen zullen doen.

Jan Blom. Jan, jij had maar weinig woorden nodig en je gaf ons het materiaal op een zilveren schaaltje. Kees en ik hoefden alleen maar te "scoren". Je werk is zeer gewaardeerd en er volgen hoop ik nog meer ideeën.

Adrie Adam. Adrie, dank je voor de duizenden dia's in honderden kleurtjes in tientallen programma's waardoor het werk ook internationaal kon worden opgevoerd.

Mijn ouders. Pap, ik dank alles aan mam en aan jou: de start in dit leven en de mogelijkheden er veel van te kunnen maken.

Eric Jan en Maria. Het is altijd zo gezellig bij jullie. Ik kom helemaal bij van al die wetenschappelijke bezigheden. Jullie zijn me erg dierbaar.

Ruben en Sjoerd. Jullie zijn het mooiste in mijn leven. Jullie foto's staan naast mijn computer en herinneren mij aan de dingen die groot geluk betekenen voor een mens; gezondheid en zorgeloosheid.

CURRICULUM VITAE

Jacquelien Pomp was born on February 28th, 1958, in Willemstad, Curaçao. She graduated from High School (Gymnasium b, Stedelijk Gymnasium in Arnhem) in 1976 and started to study Medical Biology at the University of Utrecht. In 1978 she switched to Medicine. In 1984 she became a resident at the Rotterdams Radiotherapeutisch Institute (Professor B. Van der Werff-Messing). After finishing her training she went in 1989 for one year to the Gustave Roussy Institute (Dr. A. Gerbaulet) to become skilled in brachytherapy.
Since January 1990 she is a member of the staff in the department of Radiotherapy, Delft and had the opportunity to do research at the Academic Hospital Leiden. She is the proud aunt of Ruben and Sjoerd.

LIST OF PUBLICATIONS

Papers

Pomp, J., Levendag, P.C. and van Putten, W.L. Reïrradiation of recurrent tumours of the head and neck. American Journal of Clinical Oncology, 11(5): 543-9, 1988.

Levendag, P.C, and Pomp, J.: Preliminary Experience with LDR brachytherapy for reïrradiation of advanced/recurrent head and neck cancer. Proceedings brachytherapy working conferences; 5th international selectron users meeting, The Hague, The Netherlands, 1988.

Levendag, P.C. and Pomp, J. Radiation therapy of squamous cell carcinoma of the nasal vestibule. Int. J. Radiat. Oncol. Biol. Phys.: 19. 1363-1367, 1990

Gerbaulet, A., Haie, C., Michel, G., Castaigne, D., Prade, M., Kunkler, I., Pomp, J., Masselot, J., Lhomme, C., Chassagne, D. Combined radio-surgical treatment in early invasive cervix carcinoma according to prognostic factors; Experience of the Gustave Roussy Institute. Eur. J. Gyn. Oncol, 13(3): 256-61, 1992.

Pomp, J. De rol van oncogenen in radiotherapie. Gamma (Nederlandse vereniging voor radiologische laboranten), nr. 3, pp. 65-66, 1993

Pomp, Oncogenen en radiotherapie. Medisch Journaal Delft, June 1995, pp. 86-89.

Pomp, J., Wike, J.L., Ouwerkerk, I.J.M., Hoogstraten, C., Davelaar, J. Schrier, P.I., Leer, J.W.H., Thames H.D. and Brock, W.A. Celldensity dependent plating efficiency affects outcome and interpretation of colony forming assays; Radiother. Oncol. 40: 121-125, 1996.

Pomp, J., Ouwerkerk, I.J.M., Hermans, J., Wondergem, J., Cornelisse, C.J., Leer, J.W. and Schrier, P.I.. The influence of oncogenes N-ras and c-myc on the radiation sensitivity of human melanoma cell lines; Radiat. Res.146: 374-381, 1996.

Pomp, J., Pannekoek, B.J.M. and Overdiep, S.H.. "Pleomorphic adenoma and severe tracheal obstruction" Respiratory Medicine. 889-891, 1998.

Pomp, J., Davelaar, J., Blom, J, van Krimpen, C., Quint, W.G.V. and J.J.F.M. Immerzeel, J.J.F.M: "Radiotherapy for oesophagus carcinoma: the impact of p53 on treatment outcome" Radiother. Oncol. 46: 179-184, 1998.

Pomp, J., Tervoort, M., Abels, G., van Krimpen C., en de Graaf, P.W. "Mammacarcinoom en okselkliertoiltet; een betere toekomst met minder?" Delft Medical Journal, 1998 (1)

Pomp, J., Woudstra, E.C. and H. H. Kampinga, H.H.: Pulsed Dose rate and Low Dose Rate Brachytherapy; Comparison of sparing effects between a radiosensitive and a radioresistant cell line. Radiat. Res. 151: 449-453, 1999.

Pomp, J., Blom, J., Zwinderman, A.H., van Krimpen, C.J. and Immerzeel, J.J.F.M. E-cadherin expression in oesophagus carcinoma treated by high dose radiotherapy: correlation with pre-treatment parameters and treatment outcome. J. Cancer Res. Clin. Oncol .125 (11): 641-645 1999.

Steenland, E., Leer, J.W.H., van Houwelingen, J.C. et al: The effect of a single fraction compared to multiple fractions on painful bone metastases; A global analysis of the Dutch Bone metastases Study; Radiother. Oncol. 52: 101-109, 1999.

Pomp, J., Blom, J., Zwinderman, A.H., and van Krimpen, C. P53 and radiotherapy for oesophagus carcinoma: a comparison between 4 different antibodies. Oncology Reports 7: 1075-1078, 2000

Pomp, J., Ouwerkerk, I.J.M., C. Hoogstraten, J., Wondergem, J. Davelaar, J.W.H. Leer, and P.I. Schrier: Cell cycle effects after radiation of an oncogene transfected human melanoma cell line. Oncology Reports 7: 663-667, 2000

Bookchapter

Oxford Textbook of Oncology, section 6.7. (iii) 1995: "The role of radiotherapy in the treatment of tumours of the oral cavity", A. Gerbaulet and J. Pomp.